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Identification of Molecular Markers Associated with Fungal Resistance in Norway Spruce and Common Ash

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Abstract

Trees are giants of the forests, possessing nutrient-rich tissues that make them natural targets for pathogens. In Europe, the fungal pathogens *Heterobasidion annosum s.l.* and *Hymenoscyphus fraxineus* are associated with Norway spruce and common ash, respectively. *H. annosum s.l.* causes stem and root-rot and, in economic terms, is a major pathogen of Norway spruce. *H. fraxineus*, which causes ash dieback disease, results in severe mortality of common ash, which leads to adverse ecological losses. The aim of this thesis was to use modern molecular methods to identify markers for fungal resistance in Norway spruce and common ash for early selection of superior genotypes for resistance tree breeding.

By combining genetic map-based information and transcriptome analyses, differentially expressed genes were identified that associate with resistance to *Heterobasidion parviporum* in Norway spruce. Among the candidate genes were *PaNAC04* and two of its paralogues in subgroup III-3 in the NAC family of transcription factors, a transcription factor gene *PgMYB11*, and a number of genes encoding enzyme in the biosynthesis of phenylpropanoids.

Eleven Norway spruce markers that correlated with variation in resistance to *H. parviporum* were identified in an association genetics study. Laccase *PaLAC5* was associated with the control of lesion length development and is likely to be involved in induced defence in close proximity to the *H. parviporum* infection site. *PaLAC5* may be associated with lignosuberized boundary zone formation in spruce inner bark.

Finally, one non-synonymous SNP associated with disease severity in common ash was identified in a gene encoding a subtilisin-related peptidase S8/S53 domain. The Hi-Plex-PCR amplification method demonstrated an inexpensive, time-effective method for generating data with potential for use in future tree breeding programmes. Low population differentiation between genotypes selected for disease resistance and the wild population susceptible to ash dieback indicated opportunities for further selection without significantly losing genetic diversity in the ash population.

Keywords: Forest trees, forest pathogens, MAS, induced resistance, transcriptome, association mapping, Hi-Plex-PCR, genetic diversity, tree breeding.

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Identifiering av molekylära markörer associerade med svampresistens i gran och ask

Abstrakt

Träd är skogens jättar med näringsrika vävnader som gör dem till naturliga mål för patogener. I Europa angrips granen av *Heterobasidion annosum s.l.* som orsakar stam- och rotröta och är den ekonomiskt sett viktigaste svampskadegöraren. Asken angrips sedan slutet av 1900-talet av *Hymenoscyphus fraxineus*, som orsakar askskottsjuka med omfattande träddöd, vilket också påverkar biodiversiteten negativt. Syftet med avhandlingen var att med moderna molekylära metoder identifiera markörer för svampresistens i gran och ask. Dessa kan användas för tidigt urval av bättre genotyper och i resistensförädlingsarbete.

Genom att kombinera information från genetiska kartor och transkriptomanalyser identifierades differentiellt uttryckta gener som associerar med resistens mot angrepp av *Heterobasidion parviporum* i gran. Bland kandidatgenerna fanns *PaNAC04* och två av dess paraloger i undergrupp III-3 i NAC-familjen av transkriptionsfaktorer, en transkriptionsfaktorgen *PgMYB11* samt ett flertal gener som kodar för enzym i biosyntesen av fenylpropanoider.

I en genetisk associationsstudie identifierades elva nya markörer hos gran som korrelerade med variation i resistens mot *H. parviporum*. Laccase *PaLAC5* associerade med svampens tillväxt i bark och är troligtvis involverad i inducerat försvar vid infektionsstället t.ex. i bildandet av en lignosuberiserad gränszon i innerbarken hos gran.

En ny billig, tidseffektiv metod för att generera molekylära markörer med potential att användas i framtida trädförädlingsprogram, Hi-Plex-PCR-amplifiering, utvecklades i ask och markörerna användes i en genetisk associationsstudie. En markör associerad med svårighetsgraden av askskottsjuka i ask identifierades i en gen som kodar för ett subtilisin-relaterat peptidas. Dessutom visade genotyperna att den genetiska differentieringen mellan genotyper som valts ut för sjukdomsresistens och askar provtagna i fält var låg och att man kan selektera träd för resistens mot askskottsjuka utan att väsentligt förlora den existerande genetiska mångfalden hos askpopulationen.

Nyckelord: Skogsträd, skogspatogener, MAS, inducerad resistens, transkriptom, associeringskartläggning, Hi-Plex-PCR, genetisk mångfald, trädförädling.

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Preface

लहरों से डर कर नौका पार नहीं होती,
कोशिश करने वालों की कभी हार नहीं
होती।

नन्हीं चींटी जब दाना लेकर चलती है,
चढ़ती दीवारों पर, सौ बार फिसलती
है। मन का विश्वास रगों में साहस भरता
है, चढ़कर गिरना, गिरकर चढ़ना न
अखरता है। आख़िर उसकी मेहनत
बेकार नहीं होती, कोशिश करने वालों
की कभी हार नहीं होती।

असफलता एक चुनौती है, इसे स्वीकार
करो, क्या कमी रह गई, देखो और
सुधार करो। जब तक न सफल हो,
नींद चैन को त्यागो तुम, संघर्ष का
मैदान छोड़ कर मत भागो तुम। कुछ
किये बिना ही जय जय कार नहीं होती,
कोशिश करने वालों की कभी हार नहीं
होती।

- सोहन लाल द्विवेदी

If the boat is ever afraid of the waves, it can never, ever cross the sea, only to those who try very hard. There is never, ever a defeat.

When tiny little ants carry the grain and climb, on the steep-soft walls, they fall down hundreds of times. But their mind filled fully with hope, fills up their nerves fully with courage. Falling after climbing and climbing after falling, does never make them stop from climbing, for they know that hard work never goes waste. Only to those who try very hard, there is never, ever a defeat.

Accept the fact that every failure is a challenge. Take courage from defeat and try again, till you are crowned with success. Banish sleep from your eyes at night, and do not desert and run away from this land of defeat. For without doing hard work, no success comes. Only to those who try very hard, there is never, ever a defeat

- Sohan Lal Dwivedi

Dedication

To my beloved wife Mayuri and my sweet little angel Mayra.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Chaudhary R.,*** Lundén K., Dalman K., Dubey M., Nemesio-Gorriz M., Karlsson B., Stenlid J. and Elfstrand M. Combining transcriptomics and genetic linkage based information to identify candidate genes associated with *Heterobasidion*-resistance in Norway spruce (submitted).

II Elfstrand M.,* Baison J., Lundén K., Zhou L., Vos I., Capador-Barreto H., Stein-Åslund M., Chen Z., **Chaudhary R**., Olson Å., Wu HX., Karlsson B., Stenlid J., García-Gil MR. (2020). Association genetics identifies a specifically regulated Norway spruce laccase gene, *PaLAC5*, linked to *Heterobasidion parviporum* resistance. *Plant, Cell & Environment,* 2020;10.1111/pce.13768. doi:10.1111/pce.13768

III Chaudhary R.,* Rönneburg T., Stein Åslund M., Lundén K., Brandström-Durling., Ihrmark K., Menkis A., Stener LG., Elfstrand M., Cleary M., and Stenlid J. Marker-trait associations for tolerance to ash dieback in common ash (Manuscript).

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* Corresponding author.

The contribution of Rajiv Chaudhary to the papers included in this thesis was as follows:

I Performed RNAseq bioinformatics and qPCR analysis, generated the data and contributed to the data analysis, wrote the manuscript with comments and suggestions from co-authors, and corresponded with the journal throughout the publication process.

II Generated the data for RNAseq and performed the analysis and commented on the manuscript.

III Contributed to the planning of the study, performed the experiment, generated the data and contributed to the analysis of data, wrote the manuscript with comments and suggestions from co-authors.

Abbreviations

AM	Association mapping
CCG	Confidence interval candidate gene
cDNA	Complementary DNA
CDS	Coding region sequence
cM	CentiMorgan
Dpi	Days post inoculation
E	Exclusion
FDR	False discovery rate
GLM	General linear model
IP	Infection prevention
LD	Linkage disequilibrium
LG	Linkage group
LL	Lesion length
LOD	Logarithm of the odds
LSZ	Lignosuberized boundary zone
MAF	Minor allele frequency
MAS	Marker-assisted selection
MLM	Mixed linear model
PCR	Polymerase chain reaction
PR	Pathogenesis-related protein
PVE	Phenotypic variance explained
qPCR	Real-time polymerase chain reaction
QTLs	Quantitative trait loci
RNAseq	RNA sequencing
SNP	Single-nucleotide polymorphism
SWG	Fungal growth in sapwood
WGD	Whole-genome duplication

1 Introduction

Forests have been the dominant terrestrial ecosystem of Earth for over 370 million years (Niklas, 1997) and provide important resources for humans and animals. Forests cover about 30% of the world's land area and contain 80% of the Earth's plant biomass (http://www.skogsstyrelsen.se). Forests also harbour the vast majority of the world's terrestrial life and biodiversity (Petit & Hampe, 2006). In Sweden, productive forest covers about 22.5 million hectares, which is approximately 57% of the total land mass, and the forest industry is very important in economic terms (Fransson, 2010). The forestry sector provides society with products such as construction materials, paper, bioenergy and recreation.

Pathogens and pests can negatively affect the health and biodiversity of ecologically important forest trees (Pautasso *et al.*, 2015) and may alter the balance of the ecosystem by influencing the global carbon cycle (Peltzer *et al.*, 2010; Kurz *et al.*, 2008). Fungal pathogens cause significant economic and ecological losses every year due to their impact on the forest industry and biodiversity (Bulman *et al.*, 2016; Pautasso *et al.*, 2015; Garbelotto & Gonthier, 2013; Cubbage *et al.*, 2000; Woodward, 1998).

New diseases in forest ecosystems have been reported at an increasing rate over the past century due to increased disturbance of forest ecosystems by humans, international trade and changed climatic conditions (Ramsfield *et al.*, 2016; Stenlid & Oliva, 2016; Stenlid *et al.*, 2011; La Porta *et al.*, 2008). The economic cost of forest product losses due to introduced pathogens is difficult to estimate; however, they are reported to be approximately 2.1 billion USD in the USA alone (Pimentel *et al.*, 2002). Forest diseases can also have severe ecological and social impacts, acting as threats to the public good (Stenlid *et al.*, 2011; Perrings *et al.*, 2002). For example, invasive fungal pathogens have caused a drastic decrease in the population of common ash and elm in Sweden, causing these trees be listed as threatened species in Sweden (Pihlgren *et al.*, 2010). The decline of ash and elm infected with these invasive fungal pathogens has affected

a wide range of organisms associated with these tree species, including lichens, fungi, insects and plants, causing these species to be red listed (endangered) as well (Pautasso *et al.*, 2013; Jönsson & Thor, 2012; Thor *et al.*, 2010).

Although good forest management can reduce the spread of fungal pathogens (Enderle *et al.*, 2019; Skovsgaard *et al.*, 2017; Thor *et al.*, 2006; Schmidt, 2003), management often focuses on monitoring the spread, as well as eradicating or restricting the pathogens (Sniezko & Koch, 2017; Bulman *et al.*, 2016). Stump treatment with the biocontrol agent *Phlebiopsis gigantea* (Fr.) Jülich (Holdenrieder & Greig, 1998) is an effective method of preventing the establishment of *Heterobasidion annosum sensu lato* (*s.l.*). infection at uninfected sites. However, a carryover of *H. annosum s.l.* between rotations at already infested sites is an important factor that influences the health of the new generation of trees (Oliva *et al.*, 2010). Examining and utilizing the genetic resistance of trees through breeding offers an additional powerful method of reducing pathogen damage (Bulman *et al.*, 2016; Schmidt, 2003). The term **resistance** in the narrow sense means that once a host has been infected, it restricts pathogen growth, whereas the term **tolerance** is defined as the host's ability to reduce the infection effects on fitness (Kover & Schaal, 2002). Throughout this thesis I have used the term 'resistance' in a broad sense to refer to any means by which trees may display low susceptibility to a pest or pathogen, including tolerance and avoidance mechanisms.

In this thesis, I have focussed on two tree species: Norway spruce [*Picea abies* (L.) Karst.], a gymnosperm, and common ash (*Fraxinus excelsior* L.), an angiosperm. Evolutionarily, angiosperms and gymnosperms separated about 300 million years ago (De La Torre *et al.*, 2020; Zhang *et al.*, 2004; Stewart *et al.*, 1993). In spite of this phylogenetic distance, there are reasons to believe that there are general similarities in their defence mechanism against pathogens (De La Torre *et al.*, 2020; Adomas *et al.*, 2007). Trees have a long rotation period and during their lifetime they interact with pathogens, particularly fungi, and develop constitutive or induced defences against them. Two such fungal pathogens that infect Norway spruce and common ash are *H. annosum s.l.* and *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya, respectively. *H. annosum s.l.* infections establish a pathogenic interaction with their host and damage trees in economic terms by causing stem rot, reduced incremental growth and eventually mortality, whereas *H. fraxineus* infections damage trees, causing mortality and also significant ecological losses in the ecosystem. Therefore, it is important not only to integrate disease resistance into forest breeding programmes but also to improve our understanding of the interaction between trees and pathogens. We also need strategies for early selection of superior genotypes and deployment of disease-resistant genotypes for use in tree

breeding programmes. Molecular markers can be used as a tool to enhance the selection of genotypes for disease resistance and can be applied to practical breeding programmes with high precision, reducing cost and time. In this thesis, I have focussed on identifying new candidate genes for resistance to the forest pathogens *H. annosum s.l.* and *H. fraxineus*.

1.1 Host–pathogen pathosystem

1.1.1 Norway spruce–*Heterobasidion*

Norway spruce [*Picea abies* (L.) Karst.] is an important part of the forest industry in Europe and constitutes 41% of the standing volume in Sweden (Fridman & Wulff, 2018; Loman, 2008). Norway spruce belongs to the family Pinaceae and genus *Picea*, which consists of approximately 35 species (Farjon, 1990). The natural distribution ranges across the Alps, Balkans, the Carpathians and the Pyrenees, extending in the north up to Scandinavia and merging with Siberian spruce in Northern Russia. The species complex *H. annosum s.l.*, which causes stem and root rot, is a major fungal pathogen of Norway spruce, causing economic losses to the forest industry (Garbelotto & Gonthier, 2013; Asiegbu *et al.*, 2005). The species complex has been classified into three separate European species, *H. annosum* (Fr.) Bref., *H. parviporum* Niemelä & Korhonen and *H. abietinum* Niemelä & Korhonen, based on their main host preferences, *Pinus* spp., *Picea* spp. and *Abies* spp., respectively (Dalman *et al.*, 2010; Asiegbu *et al.*, 2005).

Modern silvicultural practices facilitate the spread of *H. annosum s.l.* by creating many new wounds, such as fresh stumps. Fruiting bodies of the fungus are formed on stumps, roots or logs of dead and diseased trees (Stenlid & Redfern, 1998). Sporocarps produce airborne basidiospores that are dispersed mainly by wind. These basidiospores can infect freshly cut stumps where the mycelia of the fungus grow and spread via root contacts to neighbouring living trees (Oliva *et al.*, 2011; Redfern & Stenlid, 1998). Mycelia may stay viable on stumps or logs for many years (at least 62 years) and efficiently spread from one forest generation to the next (Stenlid, 1994). *H. annosum s.l.* enters the tree by penetrating the bark and necrotizing the phloem, cambium and sapwood (Oliva *et al.*, 2015). Once *H. annosum s.l.* reaches the heartwood, it spreads therein, creating large decay columns that can reach up to 8–12 m in spruce trunks (Vasiliauskas & Stenlid, 1998). Estimated losses to the Swedish Forestry sector of up to two million SEK every day are attributed to growth reduction and degradation of wood by *H. annosum s.l.*, with annual losses of more than 1000

million SEK per year estimated for the European forestry sector (Woodward, 1998). *H. annosum s.l.* is not only a facultative necrotroph that grows necrotrophically within the sapwood, feeding on live tissue, killing host tissue, but also, with time, becomes a saprotroph in the heartwood, feeding on dead tissue by breaking down lignin and cellulose (Olson *et al.*, 2012).

1.1.2 Common ash–*Hymenoscyphus fraxineus*

Common ash (*Fraxinus excelsior* L.) is a broad-leaved tree species of significant ecological importance in European forests (FRAXIGEN, 2005) that is planted widely for landscaping and timber production. Common ash belongs to the Oleaceae family. The genus includes four species of ash native to Europe: *F. excelsior*, *F. angustifolia*, *F. ornus* and *F. pallisiae.* Common ash is a wind-pollinated and wind-dispersed tree species that usually occurs in mixed stands with other broadleaf species, and is predominantly distributed throughout northern and central Europe, stretching from Iran to Ireland and from southern Scandinavia to northern Spain (Dobrowolska *et al.*, 2011). However, common ash is currently suffering from the ash dieback epidemic, a disease caused by the invasive ascomycete fungus *H. fraxineus* (Enderle *et al.*, 2019; Baral *et al.*, 2014), previously known as *Chalara fraxinea* (Kowalski, 2006) and *H. pseudoalbidus* (Queloz *et al.*, 2011).

The symptoms of ash dieback on common ash trees were first reported in Poland and Lithuania in the early 1990s (Timmermann *et al.*, 2011; Lygis *et al.*, 2005; Przybył, 2002). Ash dieback disease affects ash trees of all ages and often causes severe damage, resulting in high mortality rates and threatening the existence of the *F. excelsior* species over large parts of the continent (Coker *et al.*, 2019; Pautasso *et al.*, 2015; Pautasso *et al.*, 2013). Ash dieback was first noted in Sweden in 2001 (Barklund, 2005) and since then it has had a devastating effect on ash trees. Although the total percentage of ash trees in terms of the standing volume of trees in Sweden is only about 0.16% (Cleary *et al.*, 2017; Pliura *et al.*, 2017), they provide cultural and ecological services as ash trees make the perfect habitat for a number of different species of wildlife. The loss of a high proportion of ash trees will cause adverse ecological effects (Hultberg *et al.*, 2020) and reduce biodiversity (Pautasso *et al.*, 2013; Jönsson & Thor, 2012).

H. fraxineus is a necrotrophic ascomycete fungus (Stenlid *et al.*, 2017; Landolt *et al.*, 2016) that is native to Asia, where it is associated with *Fraxinus mandshurica* and *F. chinensis* (Sønstebø *et al.*, 2017; Cleary *et al.*, 2016; Gross *et al.*, 2014; Bengtsson *et al.*, 2012). *H. fraxineus* has been reported to reproduce asexually *in vitro* (Fones *et al.*, 2016). However, under natural conditions, sexual

reproduction occurs on rachises after shedding infected leaves, which then accumulate in the leaf litter (Figure 1). The leaves are the main entrance point for the pathogen into the woody parts of its main host (Cleary *et al.*, 2013; Kräutler & Kirisits, 2012; Kirisits & Cech, 2009). The fungus can also spread to woody tissues through the petiole–shoot junction (Haňáčková *et al.*, 2017). A single petiole can harbour multiple genotypes, which form black pseudosclerotia on rachises and petioles (Haňáčková *et al.*, 2017; Gross *et al.*, 2012).

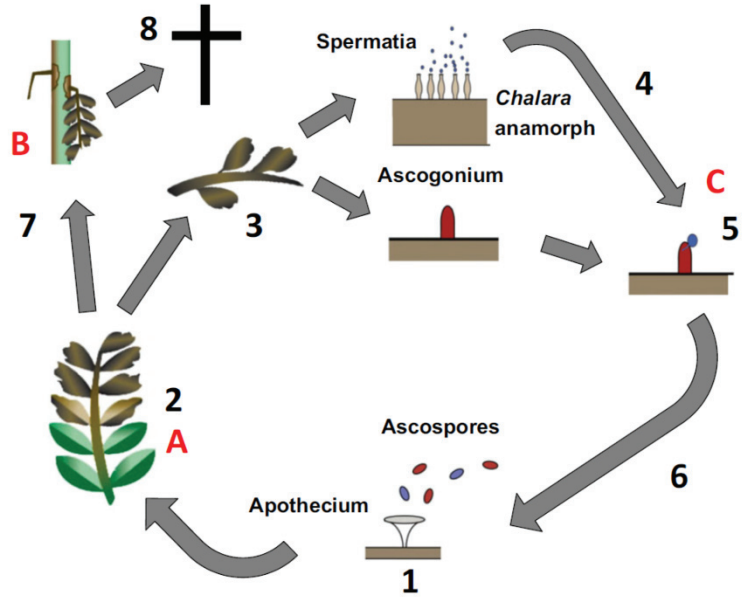

Figure 1. Overview of the life cycle of *H. fraxineus*: (1) apothecium on leaf debris from the previous year releasing male (blue) and female (red) ascospores, (2) leaf infection, (3) dead leaf falling, (4) dispersal of spermatia, (5) fertilization, (6) meiosis and ascoma development, (7) growth of the fungus into the stem, and (8) ash tree death. The red letters indicate evolutionary and ecological concepts that play an important role in ash dieback. A, the genetic diversity of common ash is crucial for resistance against *H. fraxineus*. A single leaf can face multiple infections of different strains of *H. fraxineus*. B, fitness consequences of the host defence reaction. Multiple infections of different strains can lead to competition among the strains favouring the most virulent ones. C, the sexual reproduction of *H. fraxineus* influences its genetic diversity and its evolutionary potential. Figure from Landolt et al. (2016) and reproduced with permission of the publisher.

1.2 Tree genome

1.2.1 Norway spruce

Norway spruce is diploid and has 12 (2n = 24) chromosomes and a large genome (19.6 Gb) (Nystedt *et al.*, 2013) that contains a diverse set of long terminal repeat transposable elements (70%) with a low recombination rate. Searching for genetic markers is a challenge because of the size of the genome. The number of transcribed genes makes the average distance between two neighbouring genes very large, hence, the chances of finding possible candidate genes in the vicinity of a marker associated with the trait are very low. However, during recent years, genome scans using markers (SNPs) from gene-derived sequences have become a popular method among researchers for detecting markers linked to genes (de Miguel *et al.*, 2015; Lind *et al.*, 2014; Chen *et al.*, 2012; Namroud *et al.*, 2010; Pavy *et al.*, 2006). All published genome sequence studies of members of the Pinaceae family have reported a large genome (Warren *et al.*, 2015; Neale *et al.*, 2014; Birol *et al.*, 2013; Nystedt *et al.*, 2013) and, unlike angiosperms, there is no evidence of recent whole genome duplication (WGD) in gymnosperms. A high degree of synteny and macrocollinearity has been reported within the Pinaceae family (de Miguel *et al.*, 2015; Pavy *et al.*, 2012; Pavy *et al.*, 2008; Pelgas *et al.*, 2006; Krutovsky *et al.*, 2004) for gene-based linkage maps. A predominantly out-crossing mating system has perhaps restricted conifer genomes from undergoing WGDs, thereby maintaining synteny among distantly related members of the Pinaceae family (Nystedt *et al.*, 2013; Pavy *et al.*, 2012). Because of the difficulties associated with assembling conifer DNA rich in transposable elements into scaffolds, about 30% of genes remain split across scaffolds owing to assembly fragmentation, and only a few assembled scaffolds are likely to be large enough to contain more than a single gene (Bernhardsson *et al.*, 2019; Nystedt *et al.*, 2013). The current genome assembly of Norway spruce (*P. abies* v1.0) (Bernhardsson *et al.*, 2019) covers approximately 60% of the total genome size but is still highly fragmented and consists of > 10 million scaffolds. The genome contains 66,632 gene models but there is little information available about the physical distribution of genes over the 12 linkage groups (LGs) of Norway spruce (Bernhardsson *et al.*, 2019).

1.2.2 Common ash

Common ash is diploid and has 23 (2n = 46) chromosomes (Sollars *et al.*, 2017). The genome sequence of common ash has been assembled (Kelly *et al.*, 2019; Stocks *et al.*, 2019; Sollars *et al.*, 2017). The nuclear genome is 880 Mb with

38,852 protein-coding genes. The genome has been assembled into 89,514 nuclear scaffolds, 26 mitochondrial scaffolds, and one plastid chromosome. Repetitive elements with long terminal retrotransposons are estimated to make up 35.9% of this assembly. There is evidence of whole-genome duplication events in common ash that are shared with olive (*Olea europaea*) (Sollars *et al.*, 2017). Whole genome sequencing of common ash and other ash species has also been performed to find candidate genes associated with resistance to ash dieback and the emerald ash borer (Kelly *et al.*, 2019; Stocks *et al.*, 2019).

1.3 Tree defence against pathogens

Trees being long-lived organisms face numerous challenges from pests and pathogens over their lifetime and depend on both constitutive and induced defences to restrict their attack (Kovalchuk *et al.*, 2013; Asiegbu *et al.*, 2005). Constitutive defences are non-specific and effective against a wide range of organisms as well as abiotic stressors. Trees have bark, the basic function of which is to protect nutrient-rich phloem, cambium, which is a partially undifferentiated cell layer for the radial growth of tree stems, and sapwood, which transports water and nutrients in the tree. However, sapwood is dominated by dead tissues and, hence, of the three tree tissue-types, sapwood has the least capacity to respond to pathogens (Oliva *et al.*, 2015; Johansson & Theander, 1974; Shain, 1971). The outer bark (periderm and phloem) is a natural barrier that protects the tree against biotic and abiotic stresses. Bark has lignified and suberized walls, which give strength to the tissues, making it difficult for pathogens to penetrate the outer bark (Lindberg & Johansson, 1991). When bark is damaged by a penetrating pathogen or by mechanical wounding, bark tissues attempt to seal and repair the damage by rapid necrosis of the cells closest to the wound followed by the programmed death of cells adjacent to the necrosis forming the ligno-suberized boundary (LSZ) and de-differentiation of cells next to the LSZ followed by differentiation of the wound periderm (Bodles *et al.*, 2007; Mullick, 1977). Tree bark contains phenolics and terpenoids that act as repellents and are toxic to fungal pathogens and insects (Kovalchuk *et al.*, 2013; Franceschi *et al.*, 2005). However, constitutive defences are costly and can affect the fitness of the plant (Bolton, 2009).

When the primary barriers of a host have been breached by a pathogen, inducible defences are activated when different organisms are recognized by the detection of specific microbe-associated molecular patterns (MAMPs) (Jones & Dangl, 2006). MAMPs are molecules specific to a particular group of microorganisms (e.g., peptidoglycan for bacteria or chitin for fungi) (Kovalchuk *et al.*, 2013). The defence response of plants to pathogen attack relies on the

recognition of MAMPs by pattern recognition receptors. This recognition triggers pattern-triggered immunity (PTI) by which plants are able to recognize both pathogenic and non-pathogenic invaders (Kovalchuk *et al.*, 2013). However, pathogens have evolved different effectors that contribute to their virulence, which enable them to evade the plant PTI response or even supress it, which results in the development of infection in a susceptible plant (Kovalchuk *et al.*, 2013). Effectors are proteins produced by pathogens to suppress the host defence reactions (Kovalchuk *et al.*, 2013). However, a host that is resistant to specific pathogens recognizes the effectors and activates avirulence (Avr) genes in the pathogen, which induces an effector-triggered immunity (ETI) response in the plant, which is the second line of plant defence, leading to disease resistance and often a hypersensitive cell death response (Kovalchuk *et al.*, 2013; Jones & Dangl, 2006).

In trees, the induced defence response results in a reinforcement of the cell wall through lignification and suberization (Woodward & Pearce, 1988) and the production of secondary metabolites such as terpenes, flavonoids, lignans and stilbenes (Eyles *et al.*, 2010; Witzell & Martín, 2008; Franceschi *et al.*, 2000; Lindberg *et al.*, 1992). The phenylpropanoid pathway is central to the biosynthesis of lignin, an important cell wall constituent; however, it is also activated in response to fungal colonization, and many of the lignin precursors potentially also participate in the tree defence response (Danielsson *et al.*, 2011). The induced defence response is involved in the synthesis of pathogenesis-related (PR) proteins (Kovalchuk *et al.*, 2013; Brosche *et al.*, 2010; van Loon *et al.*, 2006). PR proteins include glucanases and chitinases that break the cell wall of the invading fungi (Kovalchuk *et al.*, 2013; Eyles *et al.*, 2010; Hietala *et al.*, 2004). Some PR proteins associated with the host–pathogen interaction in tree species have been reported (Kovalchuk *et al.*, 2013; Sooriyaarachchi *et al.*, 2011; Veluthakkal & Dasgupta, 2010; Duplessis *et al.*, 2009; Elfstrand *et al.*, 2001; Sharma & Lönneborg, 1996). Furthermore, transformed plants with the highest content of *SPI 1* (spruce pathogen induced 1) displayed reduced fungal growth in the sapwood after inoculation with *H. annosum* (Elfstrand *et al.*, 2001).

1.4 Genetic diversity

Genetic diversity is important for the long-term health and survival of forest trees and for trees to evolve to resist pests and pathogens and adapt to climatic changes (Sniezko & Koch, 2017; Sollars *et al.*, 2017; Aitken *et al.*, 2008; Burdon, 2001; Namkoong, 1991). There is an overall positive correlation between population size, genetic diversity and fitness in plant species (Leimu *et al.*, 2006). Trees are

generally characterized by high genetic diversity within populations and low genetic differentiation among populations (Hamrick & Godt, 1996).

- Genetic diversity is defined as the amount of genetic variation present in a population and depends on the number and frequency of alleles that are segregating (Ingvarsson & Dahlberg, 2019).
- Gene flow is a process in which particular alleles (genes) or genotypes are transferred between close or distant geographically separated populations (McDonald & Linde, 2002).
- Population structure is defined as the amount and distribution of genetic variation within and among populations (McDonald & Linde, 2002).

The genetic diversity of Norway spruce is not under threat as Norway spruce is outbreeding, which is characterized by high gene flow and facilitated by long-distance pollen and seed dispersal with little population structure among populations. This has resulted in a large effective population size with a high level of genetic diversity within populations and a low level of genetic differentiation among populations (Bínová *et al.*, 2020; Tollefsrud *et al.*, 2009; Sperisen *et al.*, 2001; Vendramin *et al.*, 2000; Hamrick & Godt, 1996; Hamrick *et al.*, 1992). However, the adaptive potential (phenotypic variation and plasticity) may determine the vulnerability of Norway spruce (Kapeller *et al.*, 2017).

Common ash is also outcrossing and the gene flow of common ash via pollen and seed is quite high (Semizer-Cuming *et al.*, 2019), with little population structure (Tollefsrud *et al.*, 2016; Beatty *et al.*, 2015; Sutherland *et al.*, 2010; Heuertz *et al.*, 2004). However, due to ash dieback, the effective size of the ash population has declined to an extent that has caused low genetic diversity among and between ash species (Semizer-Cuming *et al.*, 2019; Lobo *et al.*, 2014; Kjær *et al.*, 2012; Pliura *et al.*, 2011). The selection pressure exerted by *H. fraxineus* on common ash is higher than for *H. annosum s.l.* on Norway spruce. Therefore, high mortality rates among common ash due to infection by *H. fraxineus* will reduce the genetic variance and could also increase its vulnerability to other pests and diseases (Evans, 2019).

1.5 Genetic variation for resistance

Plants have evolved different resistance genes specifically to defend themselves against pathogens and pests, resulting in differences in the susceptibility of the host (Namkoong, 1991). These differences between individuals could be due to a variation in a single gene (major gene or qualitative resistance) or many genes

(polygenic or quantitative resistance), each contributing a small effect to a defence response (Poland *et al.*, 2009; McDonald & Linde, 2002). In tree species, resistance variation may often appear to be quantitative due to the interaction of many genes and climatic conditions. Previous studies have reported the major gene or qualitative resistance of white pine species to white pine blister rust (Liu *et al.*, 2017; Kinloch Jr *et al.*, 1970) and of loblolly pine to fusiform rust (Wilcox *et al.*, 1996). Many studies have reported the quantitative resistance of trees to pathogens, for instance, *Pinus radiata* attacked by *Dothistroma septosporum* causing Dothistroma needle blight (Ivković *et al.*, 2010; Devey *et al.*, 2004; Chambers *et al.*, 2000), the Norway spruce–*Heterobasidion* pathosystem (Lind *et al.*, 2014; Arnerup *et al.*, 2010) and the common ash–*H. fraxineus* interaction (Sollars *et al.*, 2017; McKinney *et al.*, 2014). When one locus with large additive effects is involved, then variants of the locus that confer resistance could possibly be used in a breeding programme to produce disease-resistant trees (Burdon, 2001; Carson & Carson, 1989). Many loci controlling host resistance that each have a small effect on resistance could remain stable over many generations in the tree population in a breeding programme (Burdon, 2001; Carson & Carson, 1989). Furthermore, polygenic resistance is likely to be more stable than major gene resistance (Lindhout, 2002); it is also difficult for pathogens to break polygenic resistance (Richardson *et al.*, 2008), whereas major gene resistance is often defeated relatively quickly by pathogens (Poland *et al.*, 2009; Pinon & Frey, 2005). Quantitative resistance tends to be effective against many strains of a pathogen (McDonald & Linde, 2002).

There is evidence of phenotypic and genetic variation for resistance to *H. annosum s.l.* in Norway spruce (Chen *et al.*, 2018; Nemesio-Gorriz *et al.*, 2016; Steffenrem *et al.*, 2016; Lind *et al.*, 2014; Arnerup *et al.*, 2010; Karlsson & Swedjemark, 2006). There are no adverse correlations between resistance to *Heterobasidion* infection and growth and wood-quality traits in Norway spruce (Chen *et al.*, 2018; Steffenrem *et al.*, 2016; Karlsson & Swedjemark, 2006). Hence, selection for resistance in breeding programmes could lead to considerable gains (Chen *et al.*, 2018) without compromising other breeding achievements.

Phenotypic and genetic variation for resistance to *H. fraxineus* is also found in common ash (Harper *et al.*, 2016; Lobo *et al.*, 2014; Stener, 2013; McKinney *et al.*, 2011; Pliura *et al.*, 2011). Resistance to *H. fraxineus* in common ash (Harper *et al.*, 2016; Lobo *et al.*, 2014; Stener, 2013; McKinney *et al.*, 2011; Pliura *et al.*, 2011) has no strong negative effect on growth and survival (Lobo *et al.*, 2014; Kjær *et al.*, 2012).

Heritability is defined as the proportion of the total variance due to genetic effects. Broad-sense heritability resistance includes all genetic effects, whereas narrow-sense heritability only includes additive genetic effects that are most important to breeding programmes. The variation in genetic resistance heritability for resistance traits in Norway spruce and common ash is high enough, suggesting the possibility for early selection of superior genotypes in the presence of high infection pressure for use in resistance breeding programmes (Table 1).

Table 1. Heritability values for traits related to resistance in Norway spruce and common ash

Species	Country	Trial type	Heritability value	Reference
Norway spruce	Sweden	Progeny	h^2: 0.42	(Chen *et al.*, 2018)
Norway spruce	Norway	Progeny	H^2: 0.60	(Steffenrem *et al.*, 2016)
Norway spruce	Sweden	Progeny	H^2: 0.11	(Arnerup *et al.*, 2010)
Norway spruce	Sweden	Clonal	H^2: 0.18	(Karlsson & Swedjemark, 2006)
Common ash	Denmark	Clonal	H^2: 0.54	(McKinney *et al.*, 2011)
Common ash	Sweden	Clonal	H^2: 0.42	(Stener, 2013)
Common ash	Lithuania	Progeny	H^2: 0.57	(Pliura *et al.*, 2011)
Common ash	Denmark	Progeny	h^2: 0.52	(Kjær *et al.*, 2012)
Common ash	Lithuania	Progeny	h^2: 0.49	(Pliura *et al.*, 2011)

H^2: broad-sense heritability; h^2: narrow-sense heritability.

1.6 Artificial selection

Artificial selection may be important for generating populations with quantitative resistance (Ennos, 2015). One promising approach would be to establish seed orchards consisting of selected resistant genotypes (Kjær *et al.*, 2017; Pliura *et al.*, 2017; Stener, 2013; Kjær *et al.*, 2012; McKinney *et al.*, 2011; Pliura *et al.*, 2011). Scions collected from healthy trees and propagated by grafting to establish a resistant population would probably be a cost-effective approach (Stener, 2013), maintaining the overall genetic diversity within and among populations. Identifying and selecting superior genotypes with less susceptibility in the breeding population is also a promising strategy given the long generation time of trees (Kjær *et al.*, 2017; Pliura *et al.*, 2017; Steffenrem *et al.*, 2016; Stener, 2013; Pliura *et al.*, 2011).

1.7 Marker-assisted selection (MAS)

Due to the long generation times of forest trees, evaluating resistance properties against fungal pathogens is costly and time consuming. The development of reliable molecular markers for resistance can assist the early selection of superior genotypes (Sniezko & Koch, 2017). One marker candidate, *PaLAR3*, was identified and validated for resistance to *Heterobasidion parviporum* in Norway spruce (Nemesio-Gorriz *et al.*, 2016; Lind *et al.*, 2014). Two other reported markers in conifers, *Cr2* for resistance to the non-native invasive fungus *Cronartium ribicola* in white pine (Liu *et al.*, 2017) and *Pgβglu-1* for resistance to spruce budworm in white spruce (Mageroy *et al.*, 2015), are ready to be used for MAS of trees with improved resistance. Markers associated with resistance to myrtle rust have also been identified in *Eucalyptus*, e.g., *Ppr1* (*Puccinia psidii* resistance 1) (Mamani *et al.*, 2010; Junghans *et al.*, 2003).

An associative transcriptomics study in common ash has developed SNP and GEM (gene expression markers) associated with low susceptibility to *H. fraxineus* that showed a moderate predictive capacity (Sollars *et al.*, 2017; Harper *et al.*, 2016). Such genetic markers may enhance the process of MAS. However, for efficient MAS, several markers are needed because resistance to *H. fraxineus* in common ash is quantitative. Other methods, such as Fourier-transform infrared spectroscopy of phenolic extracts from bark tissue (Villari *et al.*, 2018) and metabolomics studies (Sambles *et al.*, 2017; Sollars *et al.*, 2017), have been used to identify resistant genotypes in ash. Such technologies may enhance selection and breeding for resistance.

MAS has not been widely implemented within tree breeding programmes, mainly owing to problems in translating quantitative trait loci (QTL) into operational MAS, i.e., the validation of potential markers (Sniezko & Koch, 2017; Neale & Kremer, 2011). Quantitative resistance is attributed to many loci with a small resistance effect. However, developing and implementing MAS for each identified locus is difficult and, hence, the desired level of resistance may not be achieved. Furthermore, markers identified in one mapping population may not be transferable to another because markers are usually only located close to genes influencing the trait and may not be actual causal variants found within the genes (Nilausen *et al.*, 2016). However, markers derived from a known gene could have the potential to associate a marker with the gene of interest (de Miguel *et al.*, 2015; Lind *et al.*, 2014; Chen *et al.*, 2012; Namroud *et al.*, 2010; Pavy *et al.*, 2006).

The two main approaches for identifying useful QTL markers for applications in tree breeding are linkage mapping and association mapping or linkage disequilibrium (LD) mapping, commonly known as genome-wide association studies (Neale & Savolainen, 2004).

1.7.1 Linkage mapping

Regions within genomes that are associated with a particular trait are known as QTLs (Collard *et al.*, 2005). Linkage mapping identifies markers inherited with a trait of interest via recombination during meiosis. Genes or markers that are tightly linked will be transmitted together from parents to progeny more frequently than genes or markers that are far apart. The closer a marker is to a QTL, the lower the chance of recombination occurring between the marker and the QTL (Collard *et al.*, 2005). It is important to find stable linkage between markers and QTLs in different environments. Although several QTLs have been identified, individual QTL explain only a small portion of the phenotypic variation (Thavamanikumar *et al.*, 2013; Khan & Korban, 2012; Neale & Kremer, 2011; Neale & Savolainen, 2004).

Several high-resolution genetic maps have been constructed for conifer trees (Bernhardsson *et al.*, 2019; Pavy *et al.*, 2017; Bartholomé *et al.*, 2015; de Miguel *et al.*, 2015; Lind *et al.*, 2014; Neves *et al.*, 2014; Pavy *et al.*, 2012). A composite map (i.e., an integrated map of different individual maps) has also been constructed for several conifer species (Pavy *et al.*, 2017; de Miguel *et al.*, 2015; Pelgas *et al.*, 2006).

1.7.2 Association mapping

Association mapping (AM) relies more on historical recombination and exploits trait variation in the mapping population instead of being limited to a single generation of parents and progenies where the number of recombination events is very small, thereby making the resolution of AM high (Baison *et al.*, 2019; Neale & Savolainen, 2004). AM is less time consuming than linkage mapping (LM) because no mapping populations need to be generated, whereas LM requires parental crosses to generate F1 or F2 populations, reducing the allelic variation in each cross (Hall *et al.*, 2010). AM is also known as linkage disequilibrium (LD mapping). LD refers to the non-random association between genetic markers (alleles) at different loci associated with QTL (see section 1.7.1). The accumulation of numerous recombination events over many generation breaks long-range linkages in natural populations and forms short stretches of high LD between loci associated with QTL (Namroud *et al.*, 2010). The association mapping approach is used for the identification of statistical associations between variations in phenotypic traits and allelic polymorphism in genes. LD decays rapidly in trees and only those markers that are tightly linked to the trait and located within the extent of LD decay demonstrate marker-trait association (González-Martínez *et al.*, 2007). AM requires the development of a very high number of markers in order to capture the short LD. Marker-trait

association may possibly be validated in one or more independent populations to identify robust markers and reduce false positives. However, the low proportion of phenotypic variation explained by individual SNPs is consistent with earlier results from LM studies (Grattapaglia *et al.*, 2018; Thavamanikumar *et al.*, 2013). LM and AM have been used to dissect the genetics of complex traits in conifers and broadleaved tree species (Table 2).

Table 2. Linkage mapping and association mapping studies in conifers and broadleaved tree species

Tree species	Mapping	Sample size	Markers	Traits	QTL/ SNP	References
Norway spruce	LM	247	686 SNP	DR	13	(Lind *et al.*, 2014)
Loblolly pine	LM	172	103 RFLP and ESTs	WPT	18	(Brown *et al.*, 2003)
Eucalyptus	LM	296	296 RFLP, RAPD, SSR, ESTs	WPT	36	(Thumma *et al.*, 2010)
Poplar	LM	343	391 AFLP, RAPD, SSR, RFLP	DR	9	(Jorge *et al.*, 2005)
Norway spruce	AM	517	178,101 SNP	WF	52	(Baison *et al.*, 2019)
Norway spruce	AM	533	373,384 SNP	DR	10	(Mukrimin *et al.*, 2018)
Poplar	AM	334	29,233 SNP	WPT	141	(Porth *et al.*, 2013)
Eucalyptus	AM	303	7,680 DArT	G & WP	18	(Cappa *et al.*, 2013)
Loblolly pine	AM	498	3,938 SNP	DR	10	(Quesada *et al.*, 2010)
White spruce	AM	492	944 SNP	WPT	21	(Beaulieu *et al.*, 2011)
Common ash	AM	1,250	3,149	DR	192	(Stocks *et al.*, 2019)

LM: linkage mapping; AM: association mapping; SNP: single-nucleotide polymorphism; RFLP: restriction fragment length polymorphism; EST: expressed sequence tag; RAPD: random amplified polymorphic DNA; SSR: simple sequence repeat; AFLP: amplified fragment length polymorphism; DArT: diversity array technology; DR: disease resistance; WPT: wood property traits; WF: wood formation; G & WP: growth and wood properties.

1.8 Sequencing strategies used in the study

1.8.1 Transcriptome sequencing

Transcriptomics sequencing (RNAseq) (Wang *et al.*, 2009b) is a method that uses next-generation sequencing platforms, e.g., Illumina, to reveal the presence and quantity of RNA in a biological sample at a specific time (Zhang *et al.*, 2018). RNAseq has become extremely popular in recent years because it uncovers information that may be missed by array-based platforms, e.g., microarrays, as no prior knowledge of the transcript sequence is needed. There is no optimal pipeline for RNAseq and researchers adopt different analysis strategies depending on the organism being studied and their research goals

(Conesa *et al.*, 2016). However, the main steps in RNAseq data analysis are experimental design, sequencing design, quality control, mapping and assembly, and differential gene expression (Conesa *et al.*, 2016). A good experimental design includes RNA extraction (e.g., mRNA), and selection of the library type, fragment size and single or paired end reads to be sequenced. Sequencing depth and number of replicates are also important for RNAseq experimental design (Conesa *et al.*, 2016). Quality-control checks should be applied to ensure both the reproducibility and reliability of the results, for instance RNAseq data should be filtered to remove adaptors and low-quality bases. The filtered read pairs are aligned to a reference genome. An established pipeline, e.g., Cufflinks, is used to assemble the transcripts followed by merging all assemblies together and, thereafter, differential expression is analysed. Transcriptome sequencing provides an approach that can be used not only to identify a large number of candidate genes but also to quantify the expression of the candidate genes (Conesa *et al.*, 2016). Gene expression during a pathogen challenge can be determined using transcriptomics. The expressed genes associated with regions under the biotic stress may possibly contribute to the resistance phenotype (Naidoo *et al.*, 2019; Nemesio-Gorriz *et al.*, 2016; Danielsson *et al.*, 2011). Transcriptome profiling of the host–pathogen interaction is important to decipher events that occur in response to pathogen invasion (Kovalchuk *et al.*, 2013). Transcriptomics profiling of the host–pathogen interaction has been studied in several forest trees, e.g., poplar (Azaiez *et al.*, 2009), Norway spruce (Dalman *et al.*, 2017; Lunden *et al.*, 2015; Danielsson *et al.*, 2011), chestnut (Barakat *et al.*, 2012), *Eucalyptus* (Meyer *et al.*, 2016a; Mangwanda *et al.*, 2015) and green ash (Lane *et al.*, 2016).

1.8.2 Exome sequencing

Exome sequencing or exome capture is a cost-effective method of sequencing targeted loci from the entire genome. This method involves probes (oligonucleotides) complementary to the target regions, e.g., exons of the genome, which are designed and hybridized to genomic DNA for sequence capture (Müller *et al.*, 2015; Grover *et al.*, 2012). Only captured DNA libraries are sequenced, which reduces the representation of sequenced regions and thereby reduces costs. Targeted sequences are captured in one hybridization reaction, and samples can be multiplexed using appropriate barcoding to capture thousands of sequences simultaneously. Exome capture has been used successfully for poplar (Zhou *et al.*, 2014), *Eucalyptus* (Dasgupta *et al.*, 2015), black spruce (Pavy *et al.*, 2016), loblolly pine (Neves *et al.*, 2014) and Norway

spruce (Elfstrand *et al.*, 2020; Baison *et al.*, 2019; Mukrimin *et al.*, 2018; Vidalis *et al.*, 2018).

1.8.3 Amplicon sequencing

Amplicon sequencing is an established method that is commonly used in molecular ecology studies. Genotyping-in-Thousands by sequencing (GT-seq) (Campbell *et al.*, 2015) and Hi-Plex2 (Hammet *et al.*, 2019) are examples of cost- and time-effective SNP genotyping methods based on custom amplicon sequencing, which uses next-generation sequencing of multiplexed PCR products, enabling the robust construction of small-to-medium panel-size libraries while maintaining low costs (Hammet *et al.*, 2019; Campbell *et al.*, 2015). Like these methods, Hi-Plex PCR for amplicon sequencing (Nguyen-Dumont *et al.*, 2013) enables the enrichment of a large number of amplicons in one single reaction tube. In multiplex sequencing, the DNA products to be sequenced in the region of interest in each sample are tagged by a unique barcode or tag (Figure 2). The sample with the tag determines from which sample the read originated, enabling the assay of multiple samples in a single sequencing run. After sequencing, the reads are sorted by detecting the unique barcode. A two-step PCR procedure is implemented in which target regions are amplified from DNA in a first round of PCR using PCR primers containing a heel sequence (linker sequence) and PCR products from this first step then serve as a template and are subjected to a second round of PCR in a successive low-cycle-number amplification adding index primers (barcode primer) or tags via the previously attached universal heel sequence.

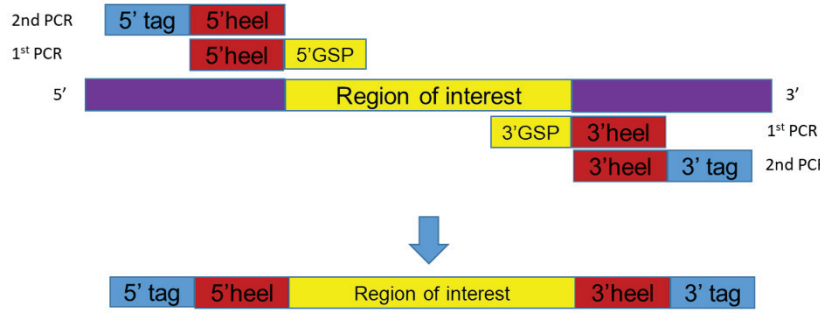

Figure 2. Schematic illustration of Hi-Plex PCR (Nguyen-Dumon et al., 2015)

2 Objectives

The overall objective of this thesis was to identify molecular markers associated with fungal resistance in two forest trees, Norway spruce and common ash. The objective had two broader aims: firstly, to identify candidate markers associated with resistance to *H. annosum s.l.* to help to reduce economic losses in Norway spruce; secondly, to identify markers associated with resistance to ash dieback to help to conserve common ash.

The specific objectives are as follows:

- To identify candidate genes associated with resistance to *H. parviporum* in the Pinaceae composite map using previously described markers in the Norway spruce linkage map with an assumption that such an approach would help to identify additional Norway spruce candidate genes associated with already-described resistance QTL (paper I).
- To evaluate the transcriptional response of these candidate genes in response to *H. parviporum* at three and seven dpi with an assumption that genes that are part of an induced defence would be upregulated in response to the pathogen (paper I).
- To identify molecular markers associated with QTL resistance to *H. parviporum* in Norway spruce using an association genetics approach and expression analysis of candidate genes *in silico* and in response to *H. parviporum*. The reasoning was that candidate genes linked to SWG are more commonly expressed in sapwood and are associated with induced defences in response to *H. parviporum* (paper II).
- To identify molecular markers for traits related to resistance to ash dieback in common ash using a modified Hi-Plex amplification method coupled with association studies (paper III).
- To investigate whether common ash genotypes selected for their resistance phenotype were genetically different from a susceptible wild population (paper III).

3 Materials and method

3.1 Plant materials

3.1.1 Norway spruce materials

In papers I and II, for the RNAseq study, six 7-year-old cuttings of each of the genotypes S21K0220126 and S21K0220184, originating from a well-studied full-sib family (S21H9820005) of Norway spruce, were used (Lind *et al.*, 2014; Arnerup *et al.*, 2011; Arnerup *et al.*, 2010).

For the real-time PCR (qPCR) experiment in paper I, cuttings of six genotypes (S21K0220263, S21K0220240, S21K0220237, S21K0220161, S21K022136 and S21K022346) from the same Norway spruce full-sib family (S21H9820005) were used (Lind *et al.*, 2014; Arnerup *et al.*, 2011; Arnerup *et al.*, 2010) and six-year-old grafted cuttings of the Norway spruce genotype S21K7820222 were used in paper II.

For phenotyping of Norway spruce, on average ten two-year-old open-pollinated progenies per mother tree were used in paper II. These were derived from 466 tested maternal trees in the Swedish breeding population, which were used for genotyping.

3.1.2 Common ash materials

In paper III, a total of 111 resistant and 215 susceptible ash genotypes were included. The material originated from several sources, combining material pre-selected for its resistant phenotype in Sweden with standing natural variation. One hundred and forty-three unrelated genotypes were assessed for disease severity and collected in the counties Uppland and Öland, representing the standing variation. Seventy plus trees grown in a seed orchards that originated

from different parts of southern Sweden were sampled (Stener, 2013). Finally, 113 ash genotypes pre-selected for their resistance to ash dieback from southern Sweden and Gotland (Menkis *et al.*, 2019) were used.

3.2 Inoculation of Norway spruce with *H. parviporum*

In papers I and II, tree branches were wounded with a 5-mm cork borer and then wooden plugs well colonized by *H. parviporum* mycelia (isolate Rb175) were inserted in the wound and sealed with Parafilm®. Control branches on the tree were also wounded and a sterile wooden plug was attached to each wound and sealed with Parafilm® (Chen *et al.*, 2018; Arnerup *et al.*, 2011).

In paper I, for the RNAseq study, bark and phloem samples were harvested at three- and seven-days post-inoculation (dpi). In paper II, for the qPCR experiment, bark and phloem samples were harvested at seven dpi. Bark surrounding the wounds and inoculation sites was cut into two sections and samples were collected at the inoculation site (A), 0–0.5 cm around the wound, and distal to the inoculation site (C), 1.0–1.5 cm from the wound. Six ramets per clone were inoculated and three inoculations per twig were performed. The bark samples were frozen separately in liquid nitrogen and stored at −80°C until further use. For the qPCR study in paper I, sampling is described in detail elsewhere (Arnerup *et al.*, 2011). Briefly, one bark and phloem sample was taken for each treatment and time point from six separate full-sib genotypes.

In paper II, for the phenotyping experiment, seedling stems were inoculated using a wooden dowel colonized by *H. parviporum* and sealed with Parafilm®. At 21 dpi, the seedlings were harvested and their phenotype was assessed by determining fungal growth in the sapwood (SWG) and lesion length (LL).

3.3 Phenotyping of resistance traits

3.3.1 Phenotyping in Norway spruce

The phenotyping method underlying paper II is described in (Chen *et al.*, 2018). In papers I and II, LL in the phloem and inner bark was estimated by measuring the lesion spread upwards and downwards from the edge of the inoculation point on the inside of the bark. SWG was estimated using the method described in (Arnerup *et al.*, 2010; Stenlid & Swedjemark, 1988). The seedlings were cut up into 5-mm discs upwards and downwards from the inoculation point and placed on moist filter papers in Petri dishes. Plates were incubated in darkness under moist conditions for one week at 21°C to induce conidia formation. After that, a

stereomicroscope was used to determine the presence or absence of *H. parviporum* conidia on each of the 5-mm discs. For each seedling, the sum of the discs where conidia were observed was multiplied by 5-mm and recorded as SWG.

3.3.2 Phenotyping in common ash

Disease severity assessments were conducted using one of three different methods in paper III. Method 1: disease severity assessment was based on a six-grade scale (Stener, 2013), ranging from 0 (no damage) to 6 (very serious damage) based on the health status of seed orchard trees. Method 2: the scoring system was based on visually monitoring the health status of the trees, where trees were classified as either resistant (0–10% crown damage) or susceptible (more than 10% crown damage) (Menkis *et al.*, 2019). Method 3: the remaining trees were phenotyped according to (Kirisits & Freinschlag, 2012). The phenotypic data from the seed orchards, Uppland and Öland were divided into two categories with a score of 1-resistant (disease severity score of 0–2.5) or 2-susceptible (greater than 2.5). All these disease severity scores were transformed into discrete unified disease scores corresponding to 1 and 2 in subsequent analyses, 1-resistant (disease severity < 10 %) and 2-susceptible (> 10%), in order to allow for marker trait association.

3.4 DNA extraction

3.4.1 Extraction of Norway spruce DNA

In paper II, total genomic DNA was extracted from 466 maternal trees using the Qiagen plant DNA extraction (Qiagen, Hilden, Germany) protocol with DNA quantification performed using Qubit® (Oregon, USA).

3.4.2 Extraction of common ash DNA

In paper III, total genomic DNA from the leaf tissue of 326 trees with a common ash genotype was isolated with CTAB buffer (Chang *et al.*, 1993) with 2% (w/v) polyvinylpolypyrrolidone added. DNA samples were purified using the NucleoSpin® gDNA Clean-up kit to remove PCR inhibitors (MACHEREY-NAGEL).

3.5 Gene model selection and primer design in common ash

In paper III, the 1000 largest contigs in the BATG-0.5 release of the ash genome (http://www.ashgenome.org/) were selected in order to create gene model-derived amplicons for genotyping. From each of the largest contigs, one or two gene models were selected based on the length of the predicted CDS of the gene models. The target sequences were downloaded to obtain the longest transcript CDS sequence from the BATG-0.5-CLCbioSSPACE genome assembly (http://www.ashgenome.org/) and collected in a Fasta file. Batchprimer3 (You *et al.*, 2008) was used to design primers with the following settings: a melting temperature of 60°–63°C, a product size of 95–105 and a primer size of 18–24. The amplicons with a product size of 97–100 bp primer pairs and the smallest Tm (melting temperature) difference and the smallest 3′ complementarity were selected. In total, 1000 amplicons were designed, with forward (ctctctatgggcagtc) and reverse (ctcgtgtctccgact) heel sequences added to each of the amplicons. In addition to this, I used 93 pairs of indexing primers with unique tags (barcode) to tag individual samples prior to pooling.

3.6 Library preparation

3.6.1 Norway spruce library preparation

In paper II, sequence capture was performed using 40,018 previously evaluated diploid probes (Baison *et al.*, 2019; Vidalis *et al.*, 2018). Probe design and sequence capture were undertaken by RAPiD Genomics (USA) as described previously (Baison *et al.*, 2019). The Illumina sequencing compatible libraries were amplified with 14 cycles of PCR and the probes were then hybridized to a pool comprising 500 ng of eight equimolar combined libraries following Agilent's SureSelect Target Enrichment System (Agilent Technologies). These enriched libraries were then sequenced to an average depth of 15x with an Illumina HiSeq 2500 system (San Diego, USA) using the 2 × 100 bp sequencing mode (Baison *et al.*, 2019).

3.6.2 Common ash library preparation

In paper III, 20 gene pools with 50 amplicons in each pool were used. Amplification by PCR was performed using 20 gene pools and 326 common ash genotypes. The ash samples were divided into five batches to allow unique tagging (barcode) to each individual genotype in the batch. The PCR conditions

used to amplify the amplicons were modified from (Nguyen-Dumont *et al.*, 2013), as described in paper III. In total, 18 libraries were prepared and separated on 1.8% agarose TAE gel. Each library was excised (approximately 150 bp) from the gel and purified using a GeneJet kit (ThermoFisher Scientific), which included isopropanol and a wash with a binding buffer step. The DNA quality of each library was quantified using a Qubit Fluorometer (Invitrogen). The 18 amplicon libraries were sequenced using the SNP&SEQ Platform (ScilifeLab, Uppsala). Sequencing was achieved by performing 150 cycles of paired-end sequencing in one run using the rapid run mode of the HiSeq 2500 system and v2 sequencing chemistry (Illumina Inc.).

3.7 Variant calling

3.7.1 Variant calling in Norway spruce

In paper II, raw reads were mapped against the *P. abies* reference genome, initial variant calling as well as the recalibration of the quality of the SNP calling applied to filter the raw variants is described in detail in (Baison *et al.*, 2019).

3.7.2 Variant calling in common ash

In paper III, the multiplexed samples were demultiplexed using a script developed in-house (http://github.com/troe27/hiplexdeplex) and implemented in Python 2.7 (https://www.python.org/) to sort reads based on their sequence tags. The data were processed using the settings described in paper III.

In paper III, the workflow for variant calling is based on the Genome Analysis Toolkit (GATK) (McKenna *et al.*, 2010). Briefly, demultiplexed Illumina sequence reads were aligned to the reference sequence (BATG-0.5-CLCbioSSPACE) (http://www.ashgenome.org/) using Burrows-Wheeler Aligner (BWA mem), version 0.7.5a (Li, 2013). Reads were then processed and Bcftools version 1.2 (Li *et al.*, 2009) was used for variant calling. All vcf files were merged with the help of Bcftools and VCFtools v0.1.12b (Danecek *et al.*, 2011) and used to filter variants. The data set was filtered for SNPs with a minor allele frequency (MAF) of 0.05 and a minimum coverage of 70%.

3.8 Population structure and association mapping analysis in common ash

In paper III, population structure was determined by performing principal component analysis (PCA) using TASSEL software, version 5.2.5 (Bradbury *et al.*, 2007), and the Bayesian clustering algorithm implemented in STRUCTURE, version 2.3.4 (Pritchard *et al.*, 2000). The Bayesian clustering algorithm estimated the number of hypothetical subpopulations (K) and the membership probability of each genotype to the subpopulations. The optimal K value was determined based on the ΔK calculated using the Structure Harvester v0.6.94 program (Earl, 2012).

In paper III, a general linear model (GLM) and a mixed linear model (MLM) implemented in TASSEL, version 5.2.5 (Bradbury *et al.*, 2007), were used to find an association between SNP markers and disease severity for ash genotypes. The GLM was constructed using SNPs as a fixed effect and a matrix of population structure as a covariate (GLM+PCA model) with a permutation test with 10,000 replicates implemented in TASSEL, version 5.2.5. The MLM model takes into consideration both population structure and relatedness (PCA+K models). The kinship matrix (K), which takes into account relatedness among genotypes as a random effect, was estimated using the pairwise kinship matrix as a covariate calculated in TASSEL, v 5.2.50 (Bradbury *et al.*, 2007). The PCA matrix was calculated incorporating the first three PCA as covariates in the model (Bradbury *et al.*, 2007). The proportion of significantly associated markers was estimated by applying a false discovery rate (FDR) procedure according to the Benjamini–Hochberg adjusted p-value ($p < 0.05$) (https://tools.carbocation.com/FDR).

3.9 Identification of conifer candidate genes associated with QTLs in Norway spruce

In paper I, markers in the Norway spruce QTL map (Lind *et al.*, 2014) were used to identify the corresponding QTL regions in the Pinaceae composite map (de Miguel *et al.*, 2015). Markers associated with the QTL regions were identified around a significant QTL LOD-peak that contained significant markers (p value < 0.05) according to a Kruskal–Wallis test in the Norway spruce linkage map (Lind *et al.*, 2014). Candidate genes between significant markers in the confidence interval were identified as confidence interval candidate genes (CCGs) in the Pinaceae composite map. Candidate genes in between the next subsequent markers outside the confidence interval were also selected and classed as putative candidate genes (PCGs). The Fasta sequences of unigene-

derived markers corresponding to genes in regions of the genome associated with resistance QTLs were downloaded from the *P. pinaster* unigene catalogue (http://www.scbi.uma.es/sustainpinedb/unigens), and the most probable Norway spruce orthologues were identified using a blastN query (E-value cutoff: 1e-3) in the Norway spruce gene catalogue (*Pabies* v1.0, www.congenie.org).

In paper II, QTLs associated with *H. parviporum* resistance were detected using the LASSO model, as described by (Li *et al.*, 2014). The position of QTLs detected in the Norway spruce genome was estimated by searching an ultra-dense genetic map (Bernhardsson *et al.*, 2019). Markers in the ultra-dense map were derived from the same probes as SNP markers holding QTLs in the Norway spruce linkage map (Lind *et al.*, 2014). The expression pattern of candidate genes associated with QTL in Norway spruce clone Z4006 and in wood were collected from expression data downloaded from the publicly available *P. abies* exAtlas (https://www.congenie.org) and NorWood v1.0 (http://norwood.congenie.org) databases, respectively.

3.10 RNA extraction, transcriptome sequencing and qPCR

3.10.1 RNA extraction

Total RNA was isolated using a CTAB extraction method (Chang *et al.*, 1993) in papers I and II. Samples were treated with DNase I (Sigma-Aldrich) to remove any genomic DNA contamination. The RNA integrity was examined using an Agilent RNA 6000 Nano kit (Agilent Technologies Inc.).

3.10.2 Transcriptome sequencing and bioinformatics analyses

In experiments performed in papers I and II, total RNA from three biological replicates of clones S21K0220126 and S21K0220184 per treatment were sequenced on the Illumina HiSeq 2500 system at the SNP&SEQ Technology Platform (SciLifeLab, Uppsala). Sequencing was performed using a HiSeq 2500 system, a paired-end 125-bp read length, and v4 sequencing chemistry.

Bioinformatics analysis for the transcriptomic studies described in papers I and II was carried out using a similar pipeline. Quality filtering was performed using Nesoni 0.97 (http://www.vicbioinformatics.com/nesoni-cookbook/index.html#). RNAseq analyses were performed using the Tophat-Cufflinks pipeline (Trapnell *et al.*, 2013; Trapnell *et al.*, 2012). The filtered read pairs were aligned to the '*Pabies1.0-all-cds*' reference gene model using Tophat, version 2.0.13 (Trapnell *et al.*, 2012).

3.10.3 qPCR analysis

In papers I and II, one µg of total RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad) in a total reaction volume of 20 µl. cDNA equivalent to 25 ng of total RNA was used as a template for each PCR reaction, using SsoFast EvaGreen Supermix (Bio-Rad). Transcript levels were quantified using an iQ™5 Multicolor Real-Time PCR detection system (Bio-Rad). The gene expression experiments were carried out using four to six biological and two technical replicates with a negative control. The relative expression was calculated from threshold cycle values (Ct) using the 2ΔΔCT-method (Livak & Schmittgen, 2001). Transcript abundance was normalized to the reference genes eukaryotic translation initiation factor 4A (elF4A) (Palovaara & Hakman, 2008) and elongation factor 1-α (ELF1α) (Arnerup *et al.*, 2011) and *Phosphoglucomutase* (Vestman *et al.*, 2011).

4 Results and discussion

4.1 Identification of markers and candidate genes for resistance to *H. parviporum* in Norway spruce with a potential role in induced defences

Candidate genes associated with resistance QTLs and also showing differential regulation in response to *H. parviporum* inoculation may be connected to the induced resistance phenotype (Nemesio-Gorriz *et al.*, 2016; Danielsson *et al.*, 2011). Genes associated with induced defences are assumed to be upregulated in response to fungal pathogens such as *H. parviporum* (Oliva *et al.*, 2015; Arnerup *et al.*, 2011; Danielsson *et al.*, 2011). For papers I and II, I compared genes that were specifically regulated in response to pathogen infection compared to wounding alone, looking at specific responses rather than a general response. Candidate genes that were not only upregulated in response to the fungus but also located in the genomic region important for controlling resistance have a stronger potential to be involved in resistance and could be developed into useful markers for resistance to support breeding programme.

The aim of the study reported in paper I was to identify novel markers associated with *H. parviporum* in Norway spruce. In paper I, the high degree of synteny and macrocollinearity within Pinaceae was used to identify additional novel markers as the Norway spruce genome was expected to harbour additional, but as yet undetected, genes (Lind *et al.*, 2014) controlling resistance to *H. parviporum* (Chen *et al.*, 2018; Hall *et al.*, 2016). A Pinaceae composite map (de Miguel *et al.*, 2015) in conjunction with RNAseq obtained from a 7-year full-sib family of Norway spruce infected by *H. parviporum* at three and seven dpi was used to identify novel candidate genes associated with QTLs for resistance to *H. parviporum* (Lind *et al.*, 2014). I identified 329 candidate genes, 83 were confidence interval candidate genes (CCGs) located between the significant

markers within the confidence interval and 246 were putative candidate genes (PCGs) located between the subsequent markers outside the confidence interval in the Pinaceae composite map (de Miguel *et al.*, 2015) (paper I). In total, 298 candidate genes were expressed, of which 124 showed differential expression (DEGs) at three and seven dpi. These DEGs could be considered as candidates for induced resistance.

The aim of the study reported in paper II was to identify markers that correlate with variation in resistance to *H. parviporum* by performing an association genetics study. Eleven novel markers for resistance to *H. parviporum* were identified, six associated with SWG and five associated with LL. The position of six of the markers was estimated using an ultra-dense genetic map of the Norway spruce genome (Bernhardsson *et al.*, 2019) (paper II). Five of the markers were independent of previously identified QTL markers as they were found in different linkage groups positioned > 30 cM away from the original QTLs (Lind *et al.*, 2014). Only one of the markers (MA_53835_9763) associating with the SWG was positioned within 4 cM of MA_14663g0020 in the confidence interval for the trait IP on LG 11 (Lind *et al.*, 2014) (paper II). The possibility that these markers target the same genomic region cannot be excluded, although it is unlikely given the short LD. A total of 14 candidate genes were identified on scaffolds bearing SNPs associated with resistance traits. Seven of the candidate genes were associated with SWG QTLs and seven were associated with LL (paper II).

4.2 DEGs in QTLs associated with resistance traits in Norway spruce

4.2.1 Sapwood growth

Fungal growth in sapwood (SWG) is a trait that reflects the capacity of the tree to restrict the spread of the pathogen in its sapwood (Lind *et al.*, 2014). In paper I, QTLs for SWG were located on LG2, LG6 and LG9 in the QTL mapping study (Lind *et al.*, 2014). Twenty-five DEGs were associated with SWG QTLs (paper 1, Figure 3). LG6 had two separate QTL regions for SWG (paper I, Figure 3). The first QTL region included six DEGs and the second QTL region comprised 10 DEGs, which were all categorized as CCGs (paper I, Figure 3).

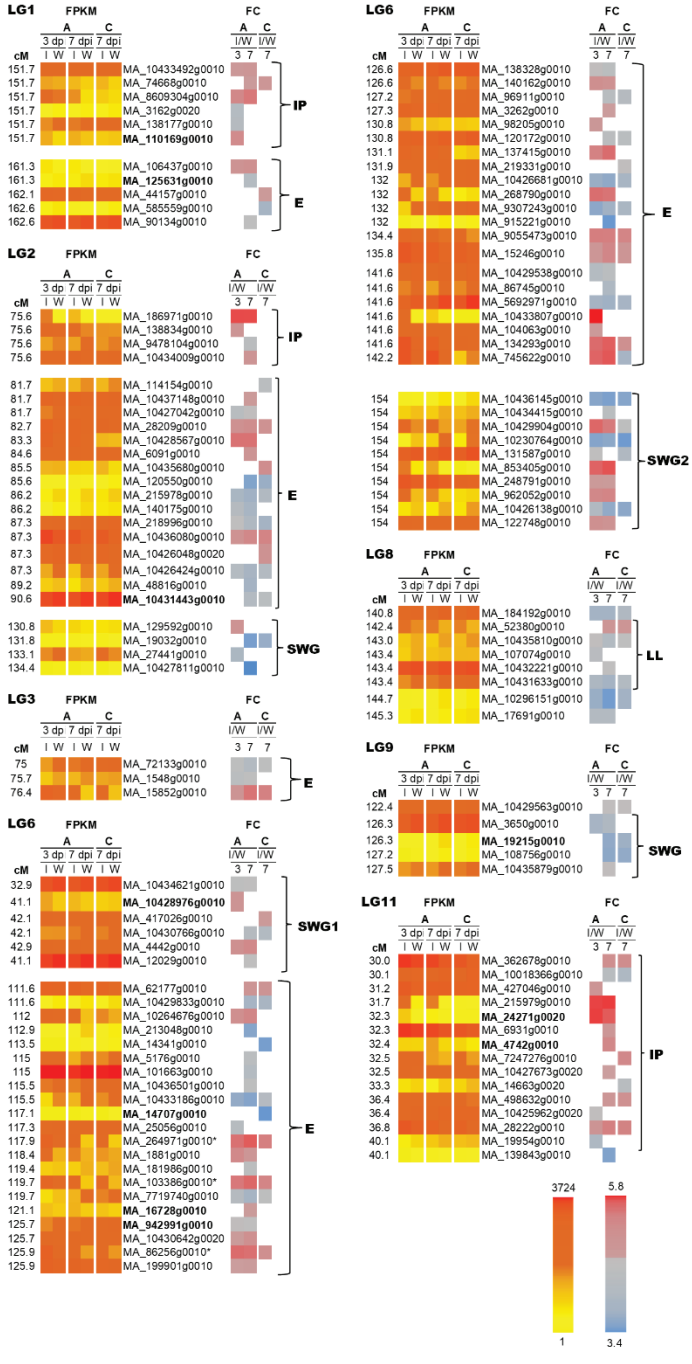

Figure 3. Heat map of the 124 differentially expressed candidate genes associated with *Heterobasidion*-resistance QTLs in Norway spruce in response to *H. parviporum*. The red to yellow colours indicate the highest to lowest FPKM (fragments per kilo base of exon model per million

reads mapped) values, respectively, at 3 and 7 dpi at the proximal (A) and distal (C) sampling sites. The red–grey–blue scale shows the highest to lowest fold change (FC, log 2 values) in *H. parviporum* inoculation compared to wounding alone. Bold font indicates candidate genes corresponding to the original QTL on the linkage groups, "E" stands for exclusion, "IP" stands for infection prevention, "LL" stands for lesion length and "SWG" stands for fungal growth in sapwood. An asterisk (*) indicates subgroup III-3 NAC TFs. cM indicates the position in centiMorgans of the marker in the Pinaceae composite map (paper I).

4.2.2 Lesion length

Lesion length or necrosis is measured as the size of the distinct necrotic tissue closest to the wound or progressing infection which provides a measure of how the defences are induced in response to pathogen invasion (Chen *et al.*, 2018; Steffenrem *et al.*, 2016; Lind *et al.*, 2014; Arnerup *et al.*, 2011; Danielsson *et al.*, 2011). Only one original marker for LL was identified on LG8. In this region, I found seven DEGs downregulated in response to *H. parviporum* except one that encodes for UDP-glucuronate 4-epimerase (MA_52380g0010), which was moderately upregulated at seven dpi (paper I, Figure 3). A *cinnamoyl-CoA reductase* (CCR) (MA_10435810g0010) identified as CCG was repressed at seven dpi both proximal and distal to the inoculation (paper I, Figure 3). The candidate gene is putatively associated with cell wall modifications. CCR is the first committed enzyme involved in the biosynthesis of monolignols (Li *et al.*, 2005; Lacombe *et al.*, 1997). CCR was downregulated and it may be possible that, this represents a redirection of resources away from the lignin biosynthesis pathway to other branches of phenolic metabolism (Ralph *et al.*, 1998) (paper I). Norway spruce, on being attacked by *H. annosum,* allocates more resources to potential antifungal low molecular weight phenolics (Danielsson *et al.*, 2011) (paper I). Previous studies in tobacco plants have reported that the downregulation of CCR activity was accompanied by the accumulation of phenolic compounds (Pinçon *et al.*, 2001; Ralph *et al.*, 1998). The downregulation of CCR gene expression in Norway spruce results in lower lignin content (Wadenbäck *et al.*, 2008).

4.2.3 Infection prevention

The infection prevention (IP) trait describes the ability of the host to stop the fungus from entering the wound upon inoculation (Lind *et al.*, 2014). QTLs associated with IP are located on LG1, LG2 and LG11 (Lind *et al.*, 2014). Twenty-five DEGs were associated with IP QTLs (paper 1, Figure 3). Four DEGs identified as CCGs were associated with IP on LG2 (Figure 3, paper I). A putative *glycosyltransferase* gene (MA_186971g0010), an orthologue of the *Arabidopsis* protein UGT85A1 that encodes a UDP-glycosyltransferase (UGT)

protein, was highly upregulated around the inoculation site at three and seven dpi (Figure 3, paper I). Glycosylation by UGTs can change phenylpropanoid solubility, stability and toxic potential, as well as influencing compartmentalization and biological activity and, hence, UGTs can affect the resistance of plants (Le Roy *et al.*, 2016). Rehman and co-workers (Rehman *et al.*, 2018) reported that the *Arabidopsis* orthologue UGT85A1 was highly upregulated in response to the necrotrophic pathogen *Alternaria brassicicola*, illustrating the importance of this UGT not only in secondary metabolite biosynthesis but also in biotic stress responses (Rehman *et al.*, 2018; Le Roy *et al.*, 2016). After inoculation of Norway spruce with *H. annosum*, aglycones increase in Norway spruce (Danielsson *et al.*, 2011).

A total of 15 DEGs were associated with the IP QTL on LG11 (Figure 3, paper I). An interesting gene on LG11 was the *PgMYB11*-like (R2R3-MYB transcription factor PgMYB11-like) gene (MA_24271g0020) associated with the original marker BT103501 in the QTL mapping study (Lind *et al.*, 2014) (paper I, Figure 3). The *PgMYB11*-like gene was highly induced at both three and seven dpi proximal to the inoculation site (paper I, Figure 3). *PgMYB11*-like has been found to be associated with adaptive trait (bud set) variation in black spruce (Prunier *et al.*, 2013). The variation in *PgMYB11*-like was associated with host defence and the timing of budset in spruce, which indicates a pleiotropic effect or tightly linked loci (Prunier *et al.*, 2013; Pelgas *et al.*, 2011) (paper I). The candidate gene MA_6931g0010 encoding a putative *caffeoyl-CoA O-methyltransferase* (*CCoAOMT*) gene showed weak upregulation at seven dpi proximal to the inoculation site (paper 1, Figure 3). *CCoAOMT1*, an enzyme in the lignin biosynthesis pathway (Ralph *et al.*, 2006) was previously shown to be involved in budworm and white pine weevil resistance in white spruce (Ralph *et al.*, 2006).

4.2.4 Exclusion

The exclusion trait is a measure of the ability of the host to hem in and exclude an invading pathogen (Lind *et al.*, 2014). QTLs associated with exclusion were located on LG1, LG2, LG3 and LG6 (Lind *et al.*, 2014). Similar to the second QTL for SWG on LG6, the exclusion QTL on LG6 had a large number of DEGs. A total of 66 DEGs were associated with exclusion QTLs (paper 1, Figure 3).

Phenylalanine ammonia lyase (PAL) is the first enzyme committed in the phenylpropanoid biosynthesis pathway (Bagal *et al.*, 2012) and common to monolignol biosynthesis and the biosynthesis of flavonoids, stilbenes and lignans (Vogt, 2010). The candidate gene MA_15852g0010 (*PaPAL3*), was located on LG3 and was upregulated at all time points (paper I, Figure 3). Two

other *PAL* genes, *PaPAL1* and *PaPAL2*, are upregulated upon *H. annosum s.l.* inoculation and wounding (Yaqoob *et al.*, 2012; Danielsson *et al.*, 2011; Koutaniemi *et al.*, 2007) (paper I). In conifers, stilbene and flavonoid monomers play a central role in the induced defence in response to pathogen and pests (Schmidt, 2005; Lieutier *et al.*, 2003; Brignolas *et al.*, 1998; Brignolas *et al.*, 1995) (paper I). In Norway spruce, flavonoids have an antimicrobial effect on *H. annosum s.l.* (Danielsson *et al.*, 2011) and *E. polonica* (Hammerbacher *et al.*, 2014; Lieutier *et al.*, 2003; Brignolas *et al.*, 1998; Brignolas *et al.*, 1995) (paper I). The stilbene astringin was negatively correlated with the depth of *H. annosum* hyphal penetration of Norway spruce bark (Lindberg *et al.*, 1992) (paper I). Stilbenes have been shown to be negatively correlated with canker pathogen in Austrian pine (Wallis *et al.*, 2008).

The QTL region for exclusion on LG6 harboured three of the previously identified Norway spruce candidate genes with similarity to subgroup III-3 NAC transcription factors (Dalman *et al.*, 2017): *PaNAC04*, MA_86256g0010 and MA_103386g0010 (paper I, Figure 3). All three candidate genes were highly upregulated in response to inoculation with *H. parviporum*, both proximal and distal to the inoculation (paper I, Figure 3). However, only *PaNAC04* was differentially regulated in S21K0220184 at seven dpi distal to the inoculation site (paper I). The upregulation of candidate genes could be associated with a redirection of resources from cell wall use to active defence in the phenylpropanoid pathway (Dalman *et al.*, 2017) (paper I). The expression pattern was comparable to that reported in a previously published phylogeny analysis of subgroup III-3 NACs (Dalman *et al.*, 2017) (paper I). Genome assembly of large and repetitive conifer genomes into scaffolds has led to assembly errors (Bernhardsson *et al.*, 2019; Nystedt *et al.*, 2013), therefore, the three highly similar candidate genes (Dalman *et al.*, 2017) may possibly correspond to one single gene located in the exclusion QTL on LG6 (paper I).

Considering the expression pattern, phylogeny analysis and complications in genome assembly, I investigated whether MA_103386g0010 represents a different gene from the previously described *PaNAC04*. A qPCR analysis of *PaNAC04* (MA_264971g0010) and MA_103386g0010 in six well-characterized Norway spruce genotypes (Arnerup *et al.*, 2011) (paper I) showed that on average *PaNAC04* was not significantly differentially expressed whereas MA_103386g0010 was differentially expressed at seven dpi in response to inoculation (Table 3). The results showed that *PaNAC04* and MA_86256g0010 probably are the same gene and separated from MA_103386p0010 in subgroup III-3 of the NAC transcription factor family (paper I). This finding is in agreement with a previous phylogenetic analysis (Dalman *et al.*, 2017). Therefore, there could be at least two NAC genes associated with this QTL.

The NAC family is one of the largest plant-specific transcription factor (TF) families, members of which are not only key regulators of developmental processes but also have been shown to control stress responses in plants (Yuan *et al.*, 2019; Olsen *et al.*, 2005). Several members of NAC are known to act as regulators of plant responses to abiotic (Puranik *et al.*, 2012; Jensen *et al.*, 2010; Wu *et al.*, 2009) and biotic stresses (Wang *et al.*, 2014; Wang *et al.*, 2009a; Wu *et al.*, 2009; Delessert *et al.*, 2005). Recent functional studies have demonstrated that a number of NAC TFs function as positive or negative regulators of plant immunity to biotrophic, hemibiotrophic or necrotrophic pathogens, (Yuan *et al.*, 2019). *PaNAC04* was responsive to *H. parviporum* inoculation in our study, similar to the response of *PaNAC03*, which was previously reported to be significantly induced against *H. parviporum* (Dalman *et al.*, 2017), indicating that *PaNAC04* is a stress-induced NAC gene, controlling flavonoid biosynthesis.

Table 3. Comparison of log2 fold change of gene expression in RNAseq and qPCR experiments

Gene	RNAseq log2 (fold change) I3 vs W3	RNAseq log2 (fold change) I7 vs W7	qPCR log2 (fold change) I3 vs W3	qPCR log2 (fold change) I7 vs W7	Annotation
MA_103386g0010	1.61*	2.81*	0.68	4.51*	NAC-transcription factor
MA_264971g0010	1.93*	2.75*	0.80	5.58	NAC-transcription factor

Comparison of expression patterns of candidate genes in RNAseq and qRT-PCR experiments. Values represent the log2 fold change in expression of candidate genes in Norway spruce when inoculated with *H. parviporum* compared to wounding alone at 3 and 7 days post inoculation (dpi). I3 and W3 indicate inoculation and wounding, respectively, at 3 dpi. I7 and W7 indicate inoculation and wounding, respectively, at 7 dpi. Asterisks (*) indicate a significantly higher induction level in response to inoculation with *H. parviporum* compared to wounding alone ($p < 0.05$) (paper I).

4.3 Norway spruce genes associated with SWG are commonly expressed in sapwood

In paper II, the expression *in silico* was evaluated using the available resources, such as NorWood and *P. abies* exATLAS databases, to gain a better understanding of the functionality of the candidate genes (paper II). It is expected that candidate genes linked to the control of SWG are involved in the reinforcement of the cell wall or in the production of secondary metabolites in sapwood (Oliva *et al.*, 2015; Stenlid & Johansson, 1987; Popoff *et al.*, 1975) (paper II). It is reasonable to hypothesize that the expression of candidate genes

linked to SWG would be more commonly expressed in the sapwood whereas candidate genes linked to LL are expressed in more peripheral tissue (paper II). Seven candidate genes MA_5978g0010, MA_5978g0020, MA_17884g0010, MA_53835g0010, MA_56128g0010, MA_18316g0010 and MA_25569g0020 were expressed in NorWood data base (paper II). Five of the candidate genes were linked to SWG. The *P. abies* exATLAS also identified all the candidate genes associated with SWG that were identified by the NorWood database. The expression of the candidate genes MA_14352g0010, MA_25569g0010, MA_97119g0010 and MA_97119g0020 associated with LL were identified in the *P. abies* exATLAS (paper II). The candidate genes identified by the NorWood database and *P. abies* exATLAS had no correlation with QTLs found for wood quality (Baison *et al.*, 2019), suggesting that the detected candidate genes linked to SWG may be associated with defence (paper II). Similarly, no negative correlations have been observed in wood quality traits and resistance to *H. parviporum* (Chen *et al.*, 2018).

4.4 *PaLAC5* gene showed the strongest induction in close proximity in response to *H. parviporum* in Norway spruce

The candidate gene associated with QTLs could affect tree resistance to *H. parviporum* infection either as part of a constitutive defence or induced defence or both (Oliva *et al.*, 2015; Arnerup *et al.*, 2011; Danielsson *et al.*, 2011). I performed an RNAseq study of transcriptional responses in bark and phloem in response to wounding and *H. parviporum* inoculation (paper II). Candidate genes MA_5978g0010, MA_5978g0020, MA_17884g0010, MA_53835g0010, and MA_56128g0010 associated with SWG showed constitutive expression at seven dpi, which we also validated by qPCR (paper II, Figure 4).

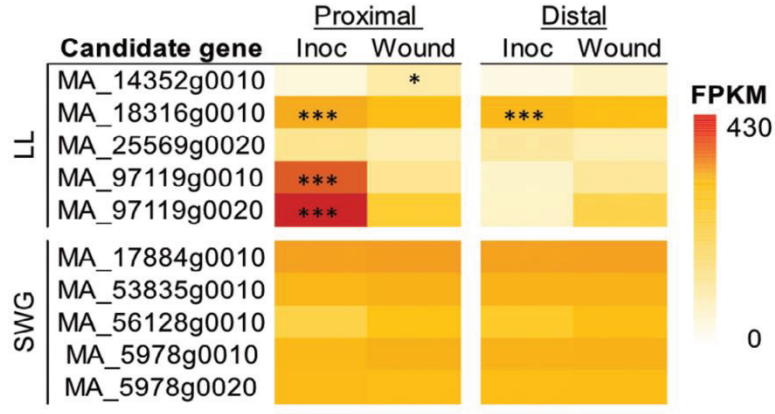

Figure 4. Expression profile of candidate genes for *H. parviporum* resistance in response to *H. parviporum* inoculation and wounding at 7 dpi proximally (0–5 mm from the inoculation site) and distally (10–15 mm from the inoculation site) in clones S21K0220126 and S21K0220184. Asterisks indicate significantly different expression levels between the inoculation treatment and the wounding control. The bar on the right indicates the FPKM values associated with the gene model. Figure from Elfstrand et al. (2020) and reproduced with permission of the publisher (paper II).

Candidate genes MA_14352g0010, MA_18316g0010, MA_97119g0010 and MA_97119g0020 associated with LL were differentially expressed at seven dpi (paper II, Figure 4). The candidate gene *PaLAC5* (MA_97119g0010 and MA_97119g0020) showed a stronger induction in response to inoculation compared with that of the wounding control proximal to the inoculation site. However *PaLAC5* was not induced, but rather down regulated distally at seven dpi (paper II, Figure 4), which was validated by qPCR (paper II). The Norway spruce laccase gene *PaLAC5* was originally isolated from a lignin-forming cell culture of Norway spruce (Koutaniemi *et al.*, 2015). RNAseq analyses of these lignin-producing Norway spruce suspension cultures indicated that *PaLAC5* is associated with the activation of stress-associated lignin production (Laitinen *et al.*, 2017). However, the transcriptional activity of *PaLAC5* is very low in sapwood and does not seem to be associated with lignification processes in wood (Blokhina *et al.*, 2019; Jokipii-Lukkari *et al.*, 2017; Laitinen *et al.*, 2017). This was also validated by qPCR, which indicated that *PaLAC5* was not significantly induced in response to *H. parviporum* in the sapwood (paper II). It could be argued that the induction of *PaLAC5* under lignin-forming conditions *in vitro* is stress associated and not directly associated with lignification processes in wood (Blokhina *et al.*, 2019; Jokipii-Lukkari *et al.*, 2017). However, the majority of *laccase* genes have been shown to have a general stress response (Koutaniemi

et al., 2007) and the *PaLAC5* expression pattern is similar to that of many other defence genes studied to date (Arnerup *et al.*, 2011; Danielsson *et al.*, 2011; Ralph *et al.*, 2006). Genes associated with lignin biosynthesis and defence-induced monolignol biosynthesis could possibly be shared in spruce (Koutaniemi *et al.*, 2007). Peroxidases and laccase have both been reported to be involved in monolignol oxidation: laccase is involved in lignin biosynthesis and peroxidases contribute to lignin formation at later stages (Zhao *et al.*, 2013). The specific isoforms of peroxidase and laccases preferentially expressed in bark suggest that they could be involved in cross-linking aromatics to form lignin-like polyphenolics (Rains *et al.*, 2018) (paper II).

The expression pattern of *PaLAC5* suggests that *PaLAC5* is associated with the formation of the lignosuberized boundary zone (LSZ) in bark adjacent to the infection site (paper II). LSZ in a tree is a constitutive defence mechanism against pathogens and is characterized by the deposition of phenolics and suberin (paper II). Previous studies have shown that the LSZ is formed during the early stages of infection to provide a barrier against fungal pathogens (Bodles *et al.*, 2007; Woodward *et al.*, 2007; Solla *et al.*, 2002; Lindberg & Johansson, 1991). *PaLAC5* associated with LL is activated in response to *H. parviporum* infection and is possibly involved in induced defence, and could also be associated with lignin production, which makes it a strong candidate for further analysis.

4.5 Hi-Plex-PCR for amplicon sequencing demonstrated a time-effective method for generating SNP markers in common ash

The ash tree has a moderately large and complex genome and, therefore, sequencing the entire genome with sufficient depth of coverage to identify reliable markers is costly. I used Hi-Plex-PCR with some modifications for amplicon sequencing to generate genomic data to identify genetic markers for resistance to ash dieback (paper III, Figure 2). Illumina HiSeq 2500 sequencing of 326 genotypes was performed with a set of 1000 new randomly selected SNP markers using a multiplex PCR approach for amplicon sequencing (Nguyen-Dumont *et al.*, 2013). The sequencing produced 655 amplified amplicons. After demultiplexing, on average, 73,261 reads were obtained per genotype (paper III). The data were filtered with a call rate of 70% and with a minor allele frequency (MAF) of 0.05, producing 40 markers in independent contigs out of 1000 SNP markers (paper III).

Despite thorough quantitative adjustments after the first and second round of amplification, 61% of total sequencing reads missed the tags. This could be due to PCR amplification bias (non-uniform amplification), poor-quality DNA,

primer dimer formation, competition from amplicons of different amplification efficiencies or the size selection purification step (Peng *et al.*, 2015). I assume that unused primers in the second round of amplification failed to add tags to the DNA. Therefore, size selection purification is a crucial step to remove unused primers (Peng *et al.*, 2015). A simple Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) analysis could have differentiated tagged samples from non-tagged samples. An enzymatic approach to degrade leftover primers might also be possible.

4.6 Low levels of differentiation between material selected for the disease resistance phenotype and the susceptible wild population in common ash

The ash dieback epidemic is likely to affect the level of resistance and genetic diversity among surviving ash species (Semizer-Cuming *et al.*, 2019; Lobo *et al.*, 2014; Kjær *et al.*, 2012; Pliura *et al.*, 2011) (paper III). The ongoing epidemic threatens to reduce the effective population size and genetic diversity to such an extent that regenerating ash populations could become impossible (Pliura *et al.*, 2017). Given the dramatic loss of genetic diversity in ash trees, one promising approach could be the selection and deployment of resistant tree genotypes to restoration sites (Kjær *et al.*, 2017; Sniezko & Koch, 2017; McKinney *et al.*, 2014; Stener, 2013; Pliura *et al.*, 2011) (paper III). However, the selection and deployment of resistant trees on a large scale may generate reduced genetic diversity, which in turn could result in reduced biodiversity and reduce the ability of populations to respond to natural selection (Black-Samuelson, 2012). It could be argued that the effective population size has already decreased to such an extent that it will compromise the remaining level of genetic diversity. However, there is a possibility that by using artificial selection to successfully regenerate ash populations (Pliura *et al.*, 2017), the genetic variation in planted stands would be maintained at comparable levels to naturally regenerated stands because the phenotypically superior trees that are artificially selected would be derived from a wide geographic range (Hallsby *et al.*, 2013). A population structure analysis was performed with 40 SNPs using both TASSEL and STRUCTURE v2.3.4 and no population structure was observed in the studied ash population (paper III). I also investigated whether the collected population of resistant ash phenotypes were genetically different from the standing natural variation. I found little population differentiation and low differentiation between genotypes selected for a resistant phenotype and that of susceptible wild populations of ash in Sweden (paper III), indicating that there are still opportunities for further selection without significantly losing existing genetic

diversity in ash (paper III). In previous studies, common ash appears to have very little population structure across Europe, showing a large and diverse population (Sollars *et al.*, 2017; Tollefsrud *et al.*, 2016; Sutherland *et al.*, 2010; Heuertz *et al.*, 2004).

4.7 Marker-trait association identifies markers associated with the resistance phenotype in common ash

In all, 249 phenotyped and genotyped ash trees from Sweden were used for a marker-trait association analysis. An MLM+PCA+K model was run with TASSEL software to investigate whether any association could be detected between SNPs and resistance to ash dieback. Two significant associations (p-value < 0.05) were detected, one of which remained statistically significant after correction for multiple testing (FDR <0.05) (paper III, Table 4). The marker-trait association identified the SNP SCONTIG5992_29927 on contig 5992, which was significantly associated with the disease severity of ash (p-value < 0.001, FDR = 0.04), explaining 5.4% of the phenotypic variance (paper III, Table 4). The SNP SCONTIG5992_29927 is located at 29,927 bp in a gene model (FRAEX38873_v2_000299890.1), and is predicted to encode a peptidase S8, subtilisin-related peptidase S8/S53 domain (paper III, Table 4). The SNP SCONTIG5992_29927 non-synonymous substitution is located in the coding region, changing the amino acid at position 658 in the predicted protein from tyrosine to aspartic acid (paper III).

Table 4. *SNP locus annotations and significance values for disease severity in ash at p < 0.05 and FDR < 0.05 using MLM*

Markers[a]	Contigs[b]	VAR[c]	Gene model[d]	Position[e]	p-value	FDR adj p-value[f]	PVE%[g]	SNP feature[h]	Annotation
SCONTIG5992_29927	CONTIG 5992	A/G	FRAEX38873_v2_000299890.1	29,927	0.001	0.048	5.4	NS	Peptidase S8, subtilisin-related|Peptidase S8/S53 domain
SCONTIG6368_39377	CONTIG 6368	C/G	FRAEX38873_v2_000311990.1	39,377	0.028	0.568	3.0	NS	Leucine-rich repeat

[a] SNP marker, the SNP name was composed of the contig number and the SNP position on the contig; [b] contig name in ash genome; [c] variation in major and minor allele frequency; [d] unique gene model in ash genome; [e]

position of SNP in the gene model; [f] adjusted p-value (false discovery rate) by Benjamini–Hochberg method; [g] percentage of phenotypic variance explained; [h] SNP variant; NS, non-synonymous SNP (paper III).

The marker-trait association analysis also detected one marginally significant association ($p < 0.05$ and FDR > 0.05), namely SCONTIG6368_39377 in contig 6368 contributing to 3.0% of PVE (paper III, Table 4). The SNP SCONTIG6368_39377 is located within the gene model FRAEX38873_v2_000311990.1 at position 39,377 bp (paper III, Table 4). The gene model encodes a leucine-rich repeat protein. The SNP SCONTIG6368_39377 is also non-synonymous and would lead to the substitution of an arginine to a glycine (paper III).

The detected SNPs explained 3.0% to 5.4% of the phenotypic variation (Table 4, paper III), which is in agreement with the phenotypic variance predicted for other forest trees and common ash for most traits, including disease resistance. Such complex traits appear to be polygenic, i.e., under the control of many genes with small-to-modest effects (Sollars *et al.*, 2017; Hall *et al.*, 2016; Namkoong, 1979). Therefore, single markers are unlikely to have very high predictive capacity. Many loci controlling disease resistance are likely to be additive and, therefore, combining multiple markers would probably increase the predictive power and assist in MAS (Sollars *et al.*, 2017; Harper *et al.*, 2016; McKinney *et al.*, 2014) (paper III).

The functions of *subtilisins* in plant–pathogen interactions are diverse and are not well understood. Several subtilisin-like serine proteases are associated with plant–microbe interactions and immunity (Figueiredo *et al.*, 2014; Antão & Malcata, 2005; Laplaze *et al.*, 2000; Tornero *et al.*, 1997) (paper III). Gene silencing of the cotton subtilisin gene *GbSBT1* reduced resistance to *Verticillium dahliae* in resistant cotton cultivars (Duan *et al.*, 2016), whereas heterologous expression of the gene enhanced the resistance of *Arabidopsis* to *Fusarium oxysporum* and *V. dahliae* infections (Duan *et al.*, 2016) (paper III) The subtilisin-like protease *SBT3* contributes to insect resistance in tomato (Meyer *et al.*, 2016b). An altered *SBT3* expression level causes changes in cell wall composition in transgenic plants, suggesting a potential involvement of this *subtilase* in the control of pectin methylesterase (PME) activity (Sénéchal *et al.*, 2014). SBT3 may be involved in the degradation or processing of proteins in the insect digestive system (Meyer *et al.*, 2016b).

5 Conclusion and future prospects

The overall aim of this thesis was to identify genetic markers in Norway spruce and common ash that correlate with variation in resistance to the fungal pathogens *H. parviporum* and *H. fraxineus*, respectively. The thesis had two broader objectives: to identify candidate markers associated with resistance to *H. annosum s.l.* to help to reduce economic losses in Norway spruce and to identify candidate genes associated with resistance to ash dieback to help to save this endangered species of ash. Different molecular methods were used to identify molecular markers associated with fungal resistance in several different Norway spruce and common ash materials. The study identified potential resistance candidates, in particular *PaNAC04*, *PaLAC5* and *subtilisin*, for functional studies and to support molecular breeding after validation, which was one of the primary objectives of this study (papers I, II and III).

In paper I, an assumption was made that genes associated with resistance QTL regions that are part of induced defence responses would be upregulated in response to the pathogen. To investigate this assumption, previously identified QTL related to *Heterobasidion*-resistance in Norway spruce was revisited (Lind *et al.*, 2014) to identify novel candidate genes associated with these QTLs in the Pinaceae composite map (de Miguel *et al.*, 2015). I combined genetic linkage map and transcriptional information to evaluate the transcriptional response of these candidate genes to *H. parviporum* at three and seven dpi. From this work, it can be concluded that such an approach can associate previously identified and novel identified candidate genes with genomic regions in Norway spruce associated with resistance QTL. Candidate genes associated with resistance QTLs that are upregulated in response *to H. parviporum* infection are possibly part of an induced defence. The allelic variation of candidate genes, especially *PaNAC04*, needs to be further studied in a future experiment. Allelic variation in the *PaNAC04* gene could explain the variation in resistance associated with QTL. Resequencing of *PaNAC04* could provide information about the allelic structure of the gene and SNP variation in *PaNAC04* and its paralogues.

In paper II, an association genetics study coupled with a transcriptomic analysis identified new potential markers for resistance to *H. parviporum* in Norway spruce. In this study, it was assumed that the candidate gene linked to SWG would be more commonly expressed in woody tissue than in bark and also that candidate genes associated with induced defences respond to *H. parviporum* inoculation. The candidate genes associated with SWG in paper II were more commonly found to be expressed in sapwood, albeit not significantly, and were also found to show more of a constitutive expression pattern than the candidate genes associated with LL. However, the transcriptional responses of the candidate genes in the inner sapwood were similar to those induced in response to *H. parviporum* in peripheral tissues, showing that the defence mechanism is induced by direct fungal contact irrespective of the tissue type (Oliva *et al.*, 2015). This could be further investigated in a future experiment by using a large number of QTLs and candidate genes for both traits. It is important to understand the nature of these interactions when carrying out resistance tree breeding to reduce the spread of the pathogen inside the tree (Oliva *et al.*, 2015). An interesting Norway spruce candidate, *laccase PaLAC5*, which is associated with lesion length, could have a potential role in lignin production and was induced in response to *H. parviporum*. Whether *PaLAC5* is involved in LSZ formation or if the genetic variation associated with *PaLAC5* influences the formation of the LSZ should be investigated. RNAi- or overexpression constructs could shed light on the role of *laccase PaLAC5* in LSZ formation and also on its role in resistance to *H. parviporum.*

In paper III, a Hi-Plex amplification method was modified and paired with association genetics studies to identify candidate markers associated with variation in resistance to *H. fraxineus.* This method proved to be a viable approach for identifying candidate markers for resistance in forest trees and identified the *subtilisin* gene for the selection of resistant ash. Next-generation sequencing technologies are improving at an enormous rate, producing numerous sequences with great depth and coverage, and the price for per base pair sequencing is going down exponentially (Stein, 2011). In my opinion, the Hi-Plex PCR amplification method has the potential to generate large amounts of genomic data to identify candidate genes in a low-cost and time-efficient way. This study also showed that selection for resistant phenotypes can be done while maintaining existing diversity in ash populations. This study also gives strong support for *ex situ* conservation strategies where resistance genotypes are collected based on their phenotypes and then molecular markers are used to survey the maintenance of genetic diversity in the collected material. This study could be improved by assessing a larger number of markers and genotypes, which would improve the associations between phenotypes and genotypes. The

SNPs in *subtilisin* are actual causal variants found within the gene influencing the level of disease severity of ash dieback, which could be validated by resequencing the *subtilisin* gene. Allelic variation in the *subtilisin* gene can explain the variation in resistance associated with disease resistance. Marker-trait associations can be validated in one or more independent populations to identify robust markers and reduce false positives (Liu *et al.*, 2017; Nemesio-Gorriz *et al.*, 2016; Mageroy *et al.*, 2015).

The work in this thesis contributes to our understanding of host–pathogen interactions. We are facing significant economic and ecological losses every year due to native as well as invasive forest pathogens. This study shows the potential use of molecular markers as an eco-friendly tool to enhance selection of resistance genotypes for tree breeding programmes with high precision, reducing cost and time. Another important aspect of this thesis is that it focusses mainly on induced defence responses, which can be durable and effective against a wide spectrum of pests and pathogens (Vallad & Goodman, 2004). Furthermore, induced responses have evolutionary and ecological advantages against pests and pathogens. An induced response is considered an eco-friendly concept for enhancing tree resistance (Eyles *et al.*, 2010). Studies have shown that induced responses in trees are highly encouraging (Arnerup *et al.*, 2013; Eyles *et al.*, 2010; Krokene *et al.*, 2008; Blodgett *et al.*, 2007; Heijari *et al.*, 2005) and the prospect of using induced responses as a future management option in forest systems is a plausible goal (Eyles *et al.*, 2010).

Forest diseases can also affect urban amenity trees, heritage trees and other trees of significant cultural value, as well as species associated with those trees. Such changes can cause severe ecological and social impacts on society (Stenlid *et al.*, 2011). The effective population size of common ash has been constantly decreasing, and will continue to decrease in the coming years due to ash dieback (Pliura *et al.*, 2017). It is also important to take other threats into account, such as the emerald ash borer (EAB), which is now spreading in European Russia (Orlova-Bienkowskaja & Volkovitsh, 2018; Straw *et al.*, 2013) and could have fatal consequences for European and North American ash. Therefore, we need a large number of genetic markers that have the potential to select for resistant genotypes, either against ash dieback or EAB or both.

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Popular science summary

Forests are the most widespread terrestrial ecological system and provide important resources for mankind. Forests are also a hub for the vast majority of the world's terrestrial life and biodiversity. Attacks by forest pathogens (native or alien) on trees not only negatively affect the health and biodiversity of the forest ecosystem but also cause large economic losses every year to the forest industry. Norway spruce represents 41% of the standing volume in Sweden. *Heterobasidion annosum sensu lato* destroys wood and reduces tree growth, which results in severe economic losses of up to two million SEK every day to the Swedish forest sector. Common ash, an ecologically important tree species, has suffered severe mortality from the epidemic ash dieback, which has resulted in reduced genetic diversity among ash species. The use of molecular markers is an eco-friendly strategy for early selection of resistant genotypes and habitat restoration. This thesis also focussed on the induced defence response to fungal pathogens because the induced defence response is considered durable and effective against a wide range of pathogens.

The main focus of the study was to identify candidate genes associated with *Heterobasidion*-resistance quantitative trait loci (QTLs) in Norway spruce. A combination of genetic and transcriptional information was used to identify a set of new differentially expressed resistance genes induced in response to a pathogen infection for future analyses. Norway spruce plants were inoculated as well as wounded, and the transcriptome of the interaction at three and seven days post infection was sequenced. The transcriptional responses of candidate genes were evaluated in response to *H. parviporum* infection associated with resistance QTLs using a gene-based composite map for Pinaceae. Several candidate genes associated with the production of antimicrobial compounds and genes known to control plant responses to their environment were identified. These genes both responded strongly to the pathogen and were found in regions of the Norway spruce genome that we know are involved in resisting *H. parviporum*.

An association genetics study paired with a transcriptomics study also identified candidate genes with an induced defence response associated with resistance to *H. parviporum*. In this study, the progeny of 466 Norway spruce trees were inoculated with *H. parviporum* and phenotyped for lesion length (LL) and fungal growth in sapwood. The expression of candidate genes associated with QTLs were analysed *in silico* and in response to *H. parviporum*. Candidate genes associated with fungal growth in sapwood are more commonly expressed in sapwood. Expression analyses showed that *PaLAC5* responds specifically and strongly near the *H. parviporum* inoculation point and but was not detected 5 mm from the inoculation point, indicating that *PaLAC5* may be associated with defensive processes just next to the progressing infection, such as lignosuberized boundary zone formation.

Another focus of the study was to identify markers for traits related to resistance to ash dieback. The adapted Hi-Plex amplification method coupled with association studies demonstrated an inexpensive time-effective method for identifying candidate genes for resistance in forest trees. Three-hundred and twenty-six ash trees from Sweden were phenotyped for disease severity and then genotyped using single-nucleotide polymorphism (SNP) markers. An association study identified one non-synonymous SNP in the gene predicted to encode for subtilisin protease. This study also showed low differentiation between genotypes selected for disease resistance and the susceptible wild population of ash. This gives an indication that further selection could take place without significantly losing genetic diversity in the ash population.

This thesis contributes to our understanding of the host–pathogen interaction and demonstrates a potential use of molecular markers against fungal pathogens.

Populärvetenskaplig sammanfattning

Skogar är de mest utbredda terrestra ekosystemen och de ger viktiga resurser för mänskligheten och de är också habitat för en rik biologisk mångfald. Skogsträdens hälsa och den biologiska mångfalden i skogarna är hotade både av inhemska och av främmande invasiva skadesvampar. Skadesvampar orsakar stora ekonomiska förluster för skogsägare och industri. I Sverige utgör granen 41% av den stående volymen av produktiv skog. Den viktigaste skadesvampen på gran i Sverige är rottickan, *Heterobasidion annosum*. Rotticka är en rotrötesvamp som både förstör trä och minskar tillväxten hos gran, vilket resulterar i ekonomiska förluster på upp till två miljoner SEK per dag för den svenska skogsindustrin. Ask, en ekologiskt och kulturhistoriskt viktig trädart har under de sista två decennierna drabbats av en ny allvarlig sjukdom, askskottsjuka som orsakas av *Hymenoscyphus fraxineus*, vilket resulterat i hög dödlighet bland askarna och hotad biologisk mångfald.

Att tidigt välja ut resistenta genotyper av träd med molekylära markörer är en miljövänlig strategi för att motverka sjukdomsspridning i skogen och för att återställa livsmiljöer. Den här avhandlingen fokuserade även på att finna markörer som är kopplade till det inducerade försvaret mot svampangrepp hos gran och ask eftersom det inducerade försvaret anses vara hållbart och effektivt mot ett brett spektrum av skadesvampar.

Studiens huvudfokus var att identifiera gener associerade med resistens mot *Heterobasidion*-rotröta hos gran. Genom att kombinera information från genetiska kartor och mätningar av geners aktivitet (uttryck) har potentiella resistensgener i det inducerade försvaret mot rotticka identifierats. Analysen byggde på infektionsförsök i gran med rotticka. Gener som var mycket aktiva i närheten av svampinfekterad vävnad identifierades. I tidigare arbeten har områden i granens arvsmassa med intressanta kandidatgener möjligen associerade med försvar mot rötangrepp identifierats och nu gjordes en mer detaljerad listning av sådana kandidater med hjälp av flera genkartor hos barrträd. De två analyserna kombinerades för att stärka indikationerna på

inducerade gener av betydelse för resistens. Flera kandidatgener associerade med produktionen av antimikrobiella föreningar och gener sådana som är kända för att kontrollera växters svar på t.ex. skadegörare och torka identifierades. Dessa gener svarade både starkt på infektion med rotticka och hittades i regioner i grangenomet som vi sedan tidigare vet är av betydelse för att reagera på infektion av rotticka.

En GWAS studie (Genome Wide Association) genomfördes också för att hitta ytterligare gener som korrelerar med resistens mot rotticka. I denna studie inokulerades avkomman från 466 träd i granförädlingsprogrammet med rotticka och tillväxten av svampen mättes i innerbark och i den vattentransporterande splintveden. Variationen i 40 000 av de 466 trädens gener kartlades med så kallade SNP markörer (Single Nucleotide Polymorphies) och markörer som samvarierade med svamptillväxt identifierades. Uttrycksmönstret av kandidatgener i och nära SNP markörerna analyserades *in silico* från tidigare undersökningar och kontrollerades med kvantitativ PCR. Kandidatgener som är förknippade med svamptillväxt i splintved uttrycks också ofta i splintved och kandidatgener associerade med innerbarken var ofta mer aktiva nära svampinfekterade vävnader i innerbarken. En gen som var särskilt starkt förknippad med rottickans angrepp i innerbark är *PaLAC5*, ett fenoloxidas, den svarade specifikt och starkt nära inokuleringspunkten men reagerade inte längre bort. Genuttrycksstudierna indikerar att *PaLAC5* kan vara förknippad med försvarsprocesser precis intill den pågående infektionen, såsom lignosuberiserad gränszonbildning, vilket är en barriärszon och ett sätt för träden att avgränsa skada i innerbark.

Ett annat fokus för avhandlingen var att identifiera markörer för egenskaper relaterade till resistens mot askskottsjuka. En ny billig, tidseffektiv metod för att generera molekylära markörer med potential att användas i framtida trädförädlingsprogram, Hi-Plex-PCR-amplifiering, utvecklades i ask och markörerna användes i en genetisk associationsstudie. En markör associerad med hur allvarligt askträden angripits av askskottsjuka identifierades i en gen som kodar för ett subtilisin-relaterat peptidas. Dessutom visade genotyperna att den genetiska differentieringen mellan genotyper som valts ut för sjukdomsresistens och askar provtagna i fält var låg och att man kan selektera träd för resistens mot askskottsjuka utan att väsentligt förlora den existerande genetiska mångfalden hos askpopulationen.

Denna avhandling bidrar till vår förståelse av värd-patogen-interaktionen och visar en potentiell användning av molekylära markörer för resistensbiologin och förädlingsarbetet mot svamppatogener hos skogsträd.

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Forest pathogens attack on trees negatively affect the health and biodiversity of economic and ecological importance trees. The aim of this thesis was to identify markers for resistance to two forest pathogens: *Heterobasidion annosum* s.l. a major pathogen, in economic terms on Norway spruce and *Hymenoscyphus fraxineus* an invasive pathogen which causes severe mortality of common ash. This thesis contributes to improve the understanding of host and pathogen interaction and demonstrate a potential use of molecular markers against fungal pathogens.

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